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# **The Role of Multiple Cell Types In the Development of Regulatory T-cells**

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## **Statement of declaration**

I Daniel John Marshall, confirm that the work presented in this thesis is my own.

Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

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# Acknowledgements

Firstly I would like to thank Ben for even giving me this project. In addition to this all his continued support and patience, when faced with looking back on it some pretty stupid questions. Obviously further acknowledgement must go to Bens role at the heart of the Natural Killers defence, without which I would have let in even more goals. I would like to thank Mandy for putting up with the 'science chat' as well as helping celebrate the highs and temper the lows, which go hand in hand with a PhD. Thanks must go to the rest of team Seddon, with particular thanks to Sim for help with experiments, mice and someone to just generally annoy. Thanks to my Mum and Dad, for all ways being on the end of the phone at any time if needed and going out of their way to help me even if I didn't realise it was good for me. Obviously not forgetting all the financial support! I would also like to mention Omar and Tinashe in the animal house for both looking after my mice and being a welcome break from the relative silence of the lab.

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## Abstract

FoxP3 expressing regulatory T-cells ( $T_{reg}$ ) are essential for preventing autoimmunity by the immune system. The dynamics and signalling requirements for  $T_{reg}$  development in the thymus are not well understood but are thought to integrate TCR, co-stimulatory and cytokine signalling. Previous studies have been hampered by the difficulty of distinguishing peripheral homeostasis from de novo thymic generation of  $T_{reg}$ . To circumvent this problem, we used mice bearing both a FoxP3 reporter allele ( $FoxP3^{GFP}$ ) and in which Zap70 expression is controlled by a Tet-inducible transgene ( $TetZap70$ ), induced by administration of antibiotic doxycycline (dox). Zap70 deficient thymocytes are arrested at the  $CD4^+CD8^+$  double positive stage of development. Induction of Zap70 expression by dox therefore restores positive selection and allows analysis of de novo  $T_{reg}$  development independently of existing peripheral  $T_{reg}$ . In timecourses of Zap70 induction of  $TetZap70$   $FoxP3^{GFP}$  mice, we found that  $T_{reg}$  develop after day 4 and remained in the thymus until day 10, at which time  $GFP^+$  Treg were first detected in peripheral lymphoid organs. To investigate the requirement for TCR signals for  $T_{reg}$  development we used a pulse of the tetracycline analog methacycline, which resulted in a tight 48h window of Zap70 induction. Remarkably, confining Zap70 expression to the first two days of thymic development was sufficient for normal development 4 days later. Using the  $TetZap70$   $FoxP3^{GFP}$  mice we also investigated the temporal requirement for  $TGF\beta$ , IL-2 and CD40 signalling during  $T_{reg}$  development. Neither  $TGF\beta$  nor CD40 signalling were required for



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de novo thymic T<sub>reg</sub> development. Using blocking antibodies and the addition of cytokine-antibody complexes revealed an essential role for IL-2 as well as a semi redundant role for IL-15. Blockade of IL-2 had no effect on induction of FoxP3 or the number of T<sub>reg</sub> induced during development. However, induction of CD25 by FoxP3<sup>+</sup> T<sub>reg</sub> was entirely IL-2 dependant. Using mixed bone marrow chimeras we show evidence supporting a hematopoietic source of thymic IL-2. We therefore propose a model of thymic T<sub>reg</sub> development in which TCR signals alone are sufficient to induce FoxP3 expression but that continued development of T<sub>reg</sub> is reinforced by IL-2.

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## List of abbreviations

<b>Ab</b>	Antibody
<b>AIRE</b>	Autoimmune Regulator
<b>BrdU</b>	Bromodeoxyuridine
<b>CNS</b>	Conserved Non-coding Sequence
<b>Cre</b>	Cre recombinase
<b>cTEC</b>	Cortical Thymic Epithelial Cells
<b>CTLA-4</b>	Cytotoxic T-lymphocytes Antigen-4
<b>CTLs</b>	Cytotoxic T-Lymphocytes
<b>DAG</b>	Diacylglycerol
<b>DCs</b>	Dendritic Cells
<b>DN</b>	Double Negative
<b>dox</b>	Doxycycline
<b>DP</b>	Double Positive
<b>FoxP3</b>	Forkhead Box P3
<b>GFP</b>	Green Fluorescent Protein
<b>GITR</b>	Glucocorticoid-Induced Tumour-necrosis factor
<b>IELs</b>	Intra-Epithelial Lymphocytes
<b>IFN</b>	Interferon
<b>Ig</b>	Immunoglobulin

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<b>IL</b>	Interleukin
<b>ITAM</b>	Immunoreceptor Tyrosine-based Activation Motif
<b>iT<sub>reg</sub></b>	Inducible Regulatory T-cell
<b>LAT</b>	Linker for Activation of T-cells
<b>MAIT</b>	Mucosal-Associated Invariant T-cell
<b>met</b>	Methycycline
<b>MHC</b>	Major Histocompatibility Complex
<b>mTEC</b>	Medullary Thymic Epithelial Cells
<b>Nf-κB</b>	Nuclear-Factor Kappa B
<b>NFAT</b>	Nuclear Factor of Activated T-cells
<b>NKT</b>	Natural Killer T-cell
<b>nT<sub>reg</sub></b>	Natural Regulatory T-cell
<b>PAMPs</b>	Pathogen Associated Molecular Patterns
<b>PRRs</b>	Pattern Recognition Receptors
<b>R26</b>	Rosa 26 reporter
<b>RAG1/2</b>	Recombination Activating Gene
<b>Ros</b>	Reactive Oxygen Species
<b>SP</b>	Single Positive
<b>TCR</b>	T-cell Receptor
<b>tDC</b>	Thymic Dendritic Cell

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<b>Tet</b>	Tetracycline
<b>TGF</b>	Transforming Growth Factor
<b>TLR</b>	Toll-Like Receptor
<b>TNF</b>	Tumour Necrosis Factor
<b>T<sub>reg</sub></b>	Regulatory T-cell
<b>TSDR</b>	T <sub>reg</sub> Specific Demethylated Region
<b>Zap70</b>	Zeta Associated Protein 70kDa

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# Chapter 1

## Introduction

### 1.1 Evolution of the immune system

#### 1.1.1 Evolution

Life began on earth approximately 3.5 billion years ago with the appearance of single-cell organisms such as eubacteria, archae-bacteria and eukaryotes. Around 600 million years ago the first multicellular organisms, metazoans, began to appear. Following this milestone in our evolutionary history, rising oxygen levels in the atmosphere led to a rapid expansion and diversification of the organisms present on earth. Since then these organisms have flourished and evolved into the plethora of organisms which populate the planet today. Increased complexity led to the evolution of dedicated immune defence systems. The next several hundred million years led to the evolution of a vast array of immunological defence strategies. Most organisms deploy these simultaneously in order to efficiently protect themselves from infection by pathogens. In response to this many micro-organisms evolved the ability to exist within hosts without triggering strong immune responses. Known as parasitism, this led to the selection of less lethal strains of micro-organisms, as host survival was evolutionary advantageous (Anon 2004). The diversification and increased complexity of the metazoans was associated with the development of a wide variety of cell surface molecules, some of which could

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behave as barriers to pathogenic infection. These therefore became the first early example of the simplest form of protection from micro-organisms, barrier formation. Relentless evolutionary pressure and diversification most likely led to the incorporation of these cell surface proteins into signalling pathways, leading to the development of the innate immune system as we know it.

### **1.1.2 Innate**

The invertebrate immune system relies on the innate immune response in order to control invading pathogens. These immune responses are surprisingly complex and compare with invertebrate immune responses in terms of complexity (Boehm 2012)(Flajnik & Pasquier 2004). Innate immune responses rely on a combination of factors to control pathogenic infection, one of which is pattern recognition receptors (PRRs). Hosts detect specific bacterial components such as peptidoglycan, lipopolysaccharide, outer-membrane proteins and a wide variety of other proteins expressed by bacteria. Janeway first termed these pathogen associated molecular patterns (PAMPs) (Medzhitov & Jr 2013). The innate immune system recognises these PAMPs using pattern recognition receptors (PRRs) such as toll-like receptors (TLRs) and peptidoglycan recognition receptors (PGRPs). Signalling through these receptors then triggers downstream signalling pathways, activating cellular and humoral effectors (Reviewed in Leulier & Lemaitre 2008)(Reviewed in Royet et al. 2011). While phagocytes ingest cells which are damaged or expressing markers associated with pathogenic bacteria, humoral defences constitute a chemical-based mechanism to destroy pathogens. This response includes the

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production of AMPs which lyse cells and ROS which oxidise lipids and damage DNA. AMPs and ROS are widely used in both vertebrates and invertebrates and appear to be conserved throughout evolution (Krasity et al. 2011)(Dowling et al. 2009)(Ausubel 2005).

### **1.1.3 Adaptive**

Vertebrates make up a small fraction of all known animal species and can be subdivided into 2 groups. The jawless vertebrates which consist of approximately 100 species of hagfish and lamprey (Janvier 2010) and the jawed vertebrates which are made up of approximately 60,000 species (Mora et al. 2011). While invertebrates rely solely on the innate immune response for clearance of pathogens, vertebrates have coevolved a second adaptive arm to their immune response. The innate immune system comprises generic receptors which recognise conserved patterns on a variety of pathogens in order to trigger an inflammatory response. The vertebrate-specific adaptive immune response by contrast depends on somatic diversification of genes which encode antigen receptors. This creates a huge repertoire of cells each expressing a slightly different antigen receptor. Interestingly the types of genes which undergo somatic diversification vary in the jawless and jawed vertebrates. Jawless vertebrates rely on leucine rich repeats containing antigen receptors termed variable lymphocyte receptors (VLRs) (Boehm 2012). These develop from a form of homologous recombination that is initiated by DNA double stranded breaks and results in non-reciprocal transfer of genetic information (Nagawa et al. 2007). Antigen receptor repertoire in jawed vertebrates however

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is the result of combinational and junctional diversity through V(D)J recombination (Schatz 2004). Junctional diversity is an important difference between the two mechanisms as it introduces non-templated sequence elements. Vertebrate-specific genetic variations were also accompanied by the development of new cell types including lymphocytes and dendritic cells (DCs) as well as the development of new specialised tissues such as the thymus and spleen. A key feature of the co-evolution of the innate and adaptive immune responses is their ability to co-operate in-order to mount effective immune responses against potentially pathogenic micro-organisms (Web et al. 2013).

#### **1.1.4 V(D)J Recombination**

Lymphocytes detect antigens using either antibodies located on the surface of B-cells, or T-cell receptors (TCR) expressed by T-cells. Antibodies are composed of 4 polypeptides, 2 heavy chains (H) and 2 light chains (L), held together by disulphide bonds (R.Porter 1973). The sequence of the N-terminus of the polypeptide chains varies greatly from one antibody to the next (V region). However the C-terminal regions remain strikingly similar (C-region) (Hilshmann & Craig 1966). This diversity in sequence ensures antibodies are able to respond to a variety of antigens. The exon which encodes the V-region of the antibody is composed of 2 or 3 individual gene segments (S. Tonegawa 1983). These are known as the variable (V), diversity (D) (only present in heavy chains) and joining (J) regions (Weigert MG, 1970)(Sakano H, 1981)(Brack et al. 1978). In order to obtain a fully functional V-region, firstly recombination of D



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and J occurs, resulting in a DJ fragment. This is followed by recombination with a V region, giving a fully functional V(D)J fragment (Reviewed in Nishana 2012).

Similar to antibodies TCRs contain 2 glycoprotein subunits each individually encoded by a somatically rearranged gene. These subunits can be either  $\alpha\beta$  or  $\gamma\delta$  pairs. At the N terminal of the TCR subunits there is a V region followed by a J region. However in TCR $\beta$  or TCR $\delta$  chains these are also interrupted by a D segment. Somatic rearrangement of the TCR chains occurs by a similar mechanism to that of antibodies, with a D-J recombination preceding that of a V-DJ recombination event on the heavy chain. TCR $\beta$  chain rearrangement always precedes TCR $\alpha$  rearrangement, occurring at the very early stages of development. Strict adherence to the chronological order of these recombination events is maintained by strict expression of the required enzymes as well as chromosomal activity of the participating gene loci (Bassing et al. 2002).

Recombination is initiated by RAG1 and RAG2 proteins. These bind to recombination signal sequence (RSS) residues which are adjacent to each sub-exon. These consist of a palindromic heptamer (CACAGTG) and an AT rich nonomer (ACAAAAACC) (Sakano H, 1979). The heptamer and nonomer regions are separated by either 12bp or 23 bp spacer regions. Generally only a 12bp RSS can combine with a 23bp RSS, helping prevent non-productive rearrangements (Sakano H, 1981). Once bound to the RSS, RAG1 and RAG2 induce a single stranded nick between the RSS and coding segment (Schlissel et al. 1993). The 3' OH on the coding strand then becomes covalently linked

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with the opposite non-coding strand phosphodiester bond, in a trans-esterification reaction. This results in a hairpin structure at the coding end and the blunt signal end (Roth et al. 1993). The signal ends remain closely associated with RAG proteins resulting in the 'post-cleavage complex' (Agrawal & Schatz 1997). The coding ends are then joined to create the exon.

## **1.2 The lymphoid compartment**

### **1.2.1 Lineage Specification in bone marrow**

The main components of the adaptive immune response are lymphocytes. There are two main types of lymphocyte, the B-cells which are bone marrow derived and the T-cells which develop in the thymus. Both B-cells and T-cells develop from multi-potent progenitors within the bone marrow. These give rise to both the myeloid compartment and multi-potent lymphoid progenitors (Pelayo et al. 2006; Pelayo et al. 2005). Common lymphoid progenitors then give rise to B-cells, T-cells and NK cells, however some dendritic cells populations are generated from the same progenitors (Baba et al. 2004). A tightly regulated network of transcription factors expressed within the lymphoid progenitors determines the fate of the cells. E2A, EBF and Pax5 expression cause a commitment to becoming a B-cell whereas GATA-3 and Notch-1 signalling specify a T-cell fate (Pelayo et al. 2006; Pelayo et al. 2005). T-lymphoid progenitors then leave the bone marrow and enter the thymus where they continue their development into fully mature T-cells.

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### 1.2.2 T-cell development

Seeding of the thymus by progenitors probably occurs as early as embryonic day 11.5 in mice. The thymus consists of 2 nodules each of which contains an outer cortical region and a central medulla. The thymus contains large numbers of developing T-cell precursors which are embedded in a network of epithelial cells known as the thymic stroma. In the post-natal thymus T-lymphoid progenitor cells enter the thymic parenchyma which are found mainly in the cortico-medullary junction, where the vasculature is well developed (Lind et al. 2001). Thymocytes then migrate out of the cortico-medullary junction to the subcapsular region of the thymic cortex (Lind et al. 2001). The chemokine receptors CXCR4, CCR7 and CCR9 are suggested to be involved in this process (Misslitz et al. 2004; Plotkin et al. 2013). Here progenitor cells are signalled to differentiate into T-cells via notch signalling. Un-committed thymocytes gradually commit to the T-cell lineage under the influence of the thymic microenvironment, resulting in the induction of T-cell receptor gene rearrangement (Dik et al. 2005). All T-cell lineages arise from thymocytes which express neither CD4 or CD8 co-receptors, known as double negative thymocytes (DNs). DN thymocytes have been further categorised depending on their expression of the markers CD25, CD44 and Kit. DN1 cells initially express CD44 and Kit but not CD25. Transition from the DN1 to the DN2 stage of development is associated with the initiation of TCR  $\delta$ ,  $\gamma$  and  $\beta$  gene rearrangement as well as the expression of CD25. The process of gene rearrangement is completed in DN3 cells which subsequently decrease expression of CD44 and Kit (Livák et al. 2013). DN3 cells which successfully

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rearrange either their TCR  $\delta$  and  $\gamma$ , or  $\beta$  chains are able to signal through their TCRs, rescuing them from apoptosis and stimulating proliferation (Michie & Zúñiga-pflücker 2002; Kruisbeek et al. 2000). Cells either form TCR  $\delta\gamma$  receptors committing them to the  $\delta\gamma$  lineage or successfully rearrange their  $\beta$ -chain which pairs with a surrogate pre-TCR $\alpha$  chain, initiating a commitment to the TCR $\alpha\beta$  lineage. Completion of the  $\beta$ -selection and  $\delta\gamma$  selection checkpoints are accompanied by CD5 and CD27 expression as well as an increase in cell size. Successful formation of a TCR defines the transition from DN3a-DN3b stages of development (Taghon et al. 2006). TCR  $\delta\gamma$  cells lineage cells remain double negative but down regulate expression of CD24 following maturation.

Cells expressing a pre-TCR $\alpha\beta$  form a complex with CD3 allowing TCR signalling, leading to proliferation, the expression of both CD4 and CD8 and the arrest of further  $\beta$ -chain rearrangement. These double positive thymocytes (DPs) make up over 80% of total thymocytes. Cells also undergo  $\alpha$  locus rearrangement immediately after pre-TCR signalling. However full scale  $\alpha$  locus rearrangement is not in place until the cells become quiescent DP cells. Cells undergo various rounds of  $\alpha$  locus rearrangement until an  $\alpha$ -chain which forms an MHC-restricted receptor when paired with the  $\beta$ -chain is obtained (Petrie 1993). The generation of a TCR $\alpha\beta$  which successfully binds MHC is thought to occur relatively infrequently, therefore most DPs express surface TCR but remain undifferentiated (Petrie, 1993). When a successfully rearranged TCR $\alpha\beta$  receptor is obtained cells must undergo positive selection in order to continue development.

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### 1.2.3 Positive and negative selection in the thymus

After successful TCR $\alpha\beta$  rearrangement, developing T-cells undergo MHC mediated positive and negative selection. Positive and negative selection in the thymus is crucial for self tolerance and therefore the avoidance of autoimmunity (Werlen 2013)(Reviewed in Starr et al. 2003). A careful balance must therefore be struck, between selecting 'useful' TCR clones which are able to recognise foreign protein epitopes. Clones which are likely to mount immune responses to self and cause autoimmunity must be purged. Positive selection of developing T-cells is initiated by TCR ligation to peptide-MHC complex's with a low affinity. The signalling strength of this interaction is crucial as peptide ligands which strongly interact with the TCR will stimulate apoptosis and negative selection. However partial interaction causes positive selection (Induce et al. 2012).

When cells progress to the DP stage of development and have undergone TCR $\alpha$  rearrangement all developmental decisions are instructed by TCR-peptide/MHC interactions. These are displayed by thymic stromal cells within the thymic microenvironment. Thymocytes whose TCR fail to interact with peptide/MHC complexes undergo death by neglect. In fact the loss of the vast majority of cells within the thymus is attributed to neglect, far exceeding that observed in negative selection (Surh & Sprent 1994). Positive selection of cells in the thymus appears to occur as a result of 2 mechanisms. Firstly TCRs present on developing thymocytes appear to have an intrinsic affinity for peptide/MHC complexes despite undergoing random rearrangements (Blackman et al. 1986; Zerrahn et al. 1997). Secondly multiple rounds of TCR $\alpha$

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rearrangement allow DP cells to generate TCRs with several distinct specificities (Petrie, Livak, Schatz, Strasser, Crispe & Shortman 1993). Despite this it is estimated that 90-95% of thymocytes are lost due to failed positive selection and death by neglect (Huesmann et al. 1991; B. K. Shortman et al. 1991).

#### **1.2.4 The signalling pathways required for thymic selection.**

Several Src and Syk family proteins have been described to be essential for proximal signalling during TCR induced positive selection. The kinase Lck is required for  $\beta$ -selection at the DN3 stage of thymic development, but is also required for positive selection (Sohn et al. 2001; Hashimoto et al. 1996). Zap70<sup>-/-</sup> mice show strongly impaired positive selection also, indicating a key role in the induction of downstream TCR signalling events. One of the main targets of Zap70 kinase activity is the adaptor protein LAT. LAT<sup>-/-</sup> mice show defects in both  $\beta$ -selection and positive selection (W. Zhang et al. 1999). This has hindered studies which have aimed to assess the role of LAT in positive and negative selection. Following phosphorylation of LAT by Zap70, LAT begins recruitment of several proteins involved in the TCR signal transduction pathway. These include GADs, SLP76, Grb2 and PLC $\gamma$ 1 (Reviewed in Wange 2000). Activation of PLC $\gamma$ 1 results in diacylglycerol (DAG) and IP3 generation. IP3 can then cause increases in intracellular Ca<sup>2+</sup> causing activation of the calcineurin pathway. The calcineurin pathway has then shown to be a crucial activator of the transcription factor NFAT. The additional target of PLC $\gamma$ 1 signalling, DAG, can then cause Ras activation via the RasGRP, class of guanine nucleotide

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exchange factors (Reviewed in Starr et al. 2003). Successful positive selection of TCR $\alpha\beta$  expressing T-cells signals proliferation, survival and CD4<sup>+</sup> or CD8<sup>+</sup> SP fate determination.

Survival and differentiation are outcomes of TCR signalling for some DP thymocytes. However not all TCR signalling events lead to this. The ligands which positively select thymocytes during development are not generally stimulatory for mature T-cells. Ligands which normally stimulate T-cells outside of the thymus cause clonal deletion when expressed intra-thymically. Known as negative selection, this is the primary mechanism of eliminating self-reactive thymocytes during development. This deletion of auto reactive thymocytes was thought to occur primarily in the medulla where medullary thymic epithelial cells (mTECs) express tissue specific antigens promiscuously. However more recent reports suggest that negative selection can occur throughout development, with DPs undergoing clonal deletion in the cortex (Stritesky 2013; Daley 2013). If developing SP thymocytes express a TCR which interacts strongly with self-peptide/MHC complexes then T-cells are stimulated to undergo apoptosis. This is discussed in more detail in section 1.4.1.

### **1.2.5 CD4/CD8 lineage decision**

Positively selected DP thymocytes have potential to become either CD4<sup>+</sup> SP or CD8<sup>+</sup> SP T-cells. Elucidating the mechanisms and signalling cues which lead to T-cells committing to either of these lineages has long been the focus of many investigations. TCR signalling has been shown be essential for commitment to

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either lineage. Various models have been proposed describing the signalling cues required for the divergence of developing thymocytes into either lineage.

A strength of signal instructional model for describing the signalling mechanisms which instruct CD4 or CD8 co-receptor expression was initially favoured. This model predicted that the strength of the TCR signal received during positive selection instructed commitment to either the CD4<sup>+</sup> SP or CD8<sup>+</sup> SP lineage (Itano & Robey 2000). As the cytoplasmic tail of CD4 binds more Lck than the CD8 co-receptor, it was proposed that the relative strength of these signals led to developing T-cells turning off either CD4 or CD8 gene expression (Itano & Robey 2000). This model was based on experiments using CD8 $\alpha$  'chimeric' co-receptors engineered to express the cytosolic domain of CD4. In vivo expression of these chimeric co-receptors resulted in MHC I restricted CD4<sup>+</sup> SP T-cells (Itano & Robey 2000). Studies involving the manipulation of the activity of the intracellular kinases Lck led to similar conclusions (Hernández-Hoyos et al. 2000). More recent investigations have analysed in more detail these studies, finding no effect on CD4/CD8 lineage choice of developing cells (Erman et al. 2006). One recent model however describes thymocytes becoming increasingly more sensitive to TCR ligation. Using mice containing an inducible tetracycline transgene of Zap70, it was found that thymocytes up-regulate Zap70 expression as DP cells mature (Saini et al. 2010). Therefore as DPs mature they become more sensitive to TCR ligation. Therefore a strong TCR signal would lead to the early development of CD4<sup>+</sup> SPs leaving weaker signalling CD8<sup>+</sup> SPs to develop later. This model suggests an important role for



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the timing and strength of TCR signalling during lineage decisions in the thymus.

More contemporary models suggest the duration of signalling directs the CD4/CD8 lineage decision. The kinetic signalling model explains the finding that CD4<sup>+</sup> CD8<sup>Lo</sup> thymocytes are lineage uncommitted precursors of both CD4<sup>+</sup> SP and CD8<sup>+</sup> SP T-cells (Brugnera et al. 2000). It has been shown that DP thymocytes upon positive selection decrease cd8 transcription irrespective of MHC restriction, signalling strength or duration of the positive selection signal. DP thymocytes then assess the ability of the thymocytes to signal in the absence of cd8 transcription. If TCR signals persist then cells maintain cd4 expression while switching off cd8, causing a commitment to the CD4<sup>+</sup> lineage. However if TCR signals cease then cells re-express cd8 and commit to a CD8 lineage. This simple mechanism allows developing cells to assess their ability to signal without the CD8 co-receptor and therefore determine appropriate co-receptor expression (Reviewed in Singer et al. 2008). For the kinetic signalling model to be true positive selection and CD4/CD8 lineage decisions must be separate events. First positive selection of developing thymocytes causes cd8 down regulation, while the presence/absence of further TCR signalling events signals commitment to the CD4 or CD8 SP lineages.

Singer and colleagues have further shown that differentiation of CD8<sup>+</sup> SP T-cells requires signalling through the IL-7 receptor (J.-hyun Park et al. 2010). In these studies the authors constitutively activate the IL-7R dependant signalling pathway. They show the development of CD8<sup>+</sup> SPs in the absence of Zap70

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signalling, concluding that development of CD8<sup>+</sup> SPs is IL-7 dependant while remaining TCR independent. However IL-7R expression has been shown to be modulated by developmental TCR-dependant signals during positive selection (Sinclair et al. 2011). Singer and colleagues therefore fail to address if IL-7 responsiveness by developing thymocytes is caused by TCR signals. Further to this, studies in Zap70<sup>-/-</sup> mice which express an adenosine deaminase driven Zap70 transgene show that TCR signalling is required throughout T-cell development. Zap70 expression is limited to DPs in these mice, consequently they fail to develop any mature CD8<sup>+</sup> SP T-cells (X. Liu et al. 2003).

#### **1.2.6 Development of T-cell populations by agonist signalling.**

TCR signals of distinct strength guide the thymic development of CD4 and CD8 lineages (discussed in section 1.2.4). However TCR signals are also thought to guide development of additional more specialised cell fates. Strong signalling through the TCR are proposed to guide commitment of NKT, CD8 $\alpha\alpha$  and MAIT cells (Mucosal associated invariant T-cells). NKT cells are a thymus dependant T-cell subset which are developmentally and functionally distinct from CD4<sup>+</sup> and CD8<sup>+</sup> T-cells. NKT cells are thought to develop from CD4<sup>+</sup> CD8<sup>+</sup> DP T-cells in the thymic cortex (Gapin et al. 2001). During development randomly rearranged NKT TCRs recognise glycolipid antigens presented by CD1d. CD1d is an MHC-class-I like molecule which is expressed by both thymic epithelium and DP thymocytes (Coles & Raulet 2003). Interestingly NKT cells seem to be selected

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by CD1d- glycolipid complexes expressed on DP thymocytes. When expression of a CD1d transgene is limited to DP thymocytes in  $Cd1d^{-/-}$  mice, the NKT cell pool remains unchanged (Wei et al. 2005). This mode of selection differs from that of conventional T-cells which are selected by the thymic epithelium. The thymic epithelium also expresses CD1d (Xu et al. 2003; Forestier et al. 2003). It therefore remains to be determined if epithelial cells also play a role in NKT cell development.

Development of mucosal associated invariant T cells (MAIT) is analogous to that of NKT cells also. MAIT cells express an invariant TCR $\alpha$  chain and similar to NKT cells, display TCRs with limited diversity. These are restricted to the MHC-like molecule MR1 (Treiner et al. 2005). MAIT cells are selected intra-thymically by hematopoietic cells which present endogenous ligands bound to MR1 (Martin et al. 2009). MAIT cell development remains poorly understood however it is thought to involve a stepwise development in the thymus followed by peripheral expansion (Martin et al. 2009). Indeed MAIT cell accumulation in the gut requires MR1 expression by B-cells and commensal flora (Treiner et al. 2003).

CD8 $\alpha\alpha$  T-cells also develop in the thymus following strong TCR signalling (Pobezinsky et al. 2012). CD8 $\alpha\alpha$  T-cells constitute a large percentage of the IELs located within the small intestine. IELs are antigen experienced cells which typically express activation markers such as CD44 and CD69. They function as the first line of defences against invading pathogens located within the gut. They are typically heterogeneous and vary widely in their distribution within the

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small and large intestine (Reviewed in Cheroutre et al. 2011). A commitment to the CD8 $\alpha\alpha$  T-cell lineage is thought to result from chronic TCR signalling during thymic development. Thymocytes which are prevented from undergoing clonal deletion by either over-expressing anti-apoptotic factors or removing CD28 co-stimulation are diverted to become anergic CD4 $^{-}$  CD8 $^{-}$  DN thymocytes. These DN TCR $\alpha\beta$  T-cells then preferentially migrate to the small intestine where they differentiate into CD8 $\alpha\alpha$  IELs.

An alternative fate for thymocytes which undergo strong thymic selection signals is commitment to becoming an nT $_{reg}$ . Increased avidity for MHC/self-peptide complexes displayed by the thymic stroma during thymic selection has been shown to be essential for nT $_{reg}$  differentiation. It has also been suggested that T $_{reg}$  development requires an IL-2 signal for FoxP3 expression, which subsequently instructs a commitment to the T $_{reg}$  lineage allowing T $_{reg}$  to dampen immune responses in the periphery (discussed in 1.4.2).

### **1.3. Peripheral T-cell subsets**

Upon response to an acute infection antigen specific T-cells undergo clonal expansion and differentiate into effector T-cells. Many of these cells can then enter the blood and migrate to the area of infection.

#### **1.3.1 CD8 $^{+}$ subset**

Infections with viruses are focussed primarily on the type I response involving CD8 $^{+}$  SP cytotoxic T-lymphocytes (CTLs). CD8 $^{+}$  SP T-cells recognise peptide

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fragments from cytosolic pathogens bound to MHC class I molecules on infected cells. These interactions promote differentiation of naïve CD8<sup>+</sup> SP T-cells into CTLs (Reviewed in Kaech & Cui 2012). On interaction of a CTL with a target cell there is direct exocytosis of the CTL granules into the extracellular space between the two cells. CTL granules contain both perforin and granzymes proteins, which together cause target cell death. Perforin is a 67 kDa multi-domain protein which oligomerises on target cells, forming pores in the target cell surface (Tschopp J, Masson D 1986). Subsequent entry of granzyme A and B into the target cell induces cell death. Cell death is initiated via cleavage of pro-caspase molecules, resulting in the release of cytochrome c and the initiation of the intrinsic cell death pathway (Reviewed in Barry & Bleackley 2002). CTLs express high levels of surface FASL (CD96) and are therefore able to induce cell death via the extrinsic cell death pathway. Upon engagement with FAS, expressed on the target cell surface, Pro-caspase-8 is recruited via FADD adapter protein (Fas-associated death domain protein). Caspase 8 is then cleaved and activated initiating apoptosis via caspase 9 and recruitment of proapoptotic proteins such as BID and BAX (Barry & Bleackley 2002). As well as killing target cells, upon activation CTLs secrete cytokines such as IFN $\gamma$  and TNF.

### **1.3.2 CD4<sup>+</sup> subset**

CD4<sup>+</sup> effector subsets were initially described by Mossman and Coffman in the 1980s in the Th1 and Th2 paradigm. Th1 cells were described as a subset of mature CD4<sup>+</sup> SPs which secreted predominantly IL-2 and IFN $\gamma$ . While a second

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subset of cells, later named Th2 cells helped B cells secrete IgE by producing a specific B cell stimulating factor, later called IL-4 (Carty 1986; Cher & Mosmann 1987). Blocking of Th2 mediated IgE secretion by the Th1 factor IFN $\gamma$  later gave rise to the Th1/Th2 paradigm, in which each subset exert distinct immune responses while regulating the other subset. Th1 cells promote cell-mediated responses to cells infected with viruses and bacteria, secreting IFN $\gamma$ , TNF $\alpha$  and IL-2. Th1 cells have also been shown to support some B-cell help, mainly causing IgG2a secretion in mice (Mosmann TR 1989)(Coffman 2006). Th2 cells have been shown to produce IL-4, IL-5 and IL-13 signature cytokines and provide help in IgE secretion by B-cells for clearance of extracellular pathogens. Th2 cells have also been implicated in various forms of allergy (Mosmann TR 1989)(Coffman 2006). Further to the seminal work carried out by Mosman and Coffman, a diverse range of CD4<sup>+</sup> effector states are now known to exist. These include the discussed Th1 and Th2 lineages as well as the more recently discovered Th17, Th9, Th22, T<sub>reg</sub> T<sub>R1</sub> and T<sub>FH</sub> subsets. Th17 cells are a more recently identified class of effector T-cells that have been shown to produce IL-17 (A-F) as well as IL-21 and IL-22. IL-17 production is implicated in various autoimmune disease such as multiple sclerosis, rheumatoid arthritis, psoriasis, IBD and allergic responses (Langrish et al. 2005). Th9 cells are an even more recent helper T-cell subset to be described. These cells generate high levels of IL-9 and IL-10. They share common features with Th2 cells however are distinct with their IL-9 production (Awasthi et al. 2009; Veldhoen et al. 2008). Regulatory T-cells oppose the activity of all of these pro-inflammatory T-effector cell subsets by dampening down immune responses.

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## **1.4 Tolerance**

### **1.4.1 Central tolerance**

Recent studies have suggested that as many as six times more cells undergo negative selection than complete positive selection. It is thought that approximately 75% of these negatively selected cells are deleted at the DP stage of development (Stritesky et al. 2013). Both the thymic cortex and medulla are therefore thought to be able to contribute to central tolerance via the deletion of auto-reactive cells. The thymus provides a an environment which is critical to the establishment of tolerance (Reviewed in Kyewski & Klein 2006)(Reviewed in Anderson et al. 2007). In the medullary region of the thymus mTECs and thymic dendritic cells (tDCs), display tissue specific self-antigens and together support the negative selection of self-reactive thymocytes. The expression of these tissue specific antigens by mTECs is thought in part to be due to the transcriptional factor Autoimmune Regulator (AIRE) (M. S. Anderson et al. 2002). In both humans and mice AIRE deficiency has been shown to lead to a failure in the establishment of central tolerance through negative selection (Blechsmidt et al. 1999; Shuichi Asakawa 1997). Thymic DCs cross present mTEC derived tissue specific antigens, while a small proportion of thymic DCs migrate into the thymus from the periphery. This allows thymic import of self-antigens from peripheral tissues (Bonasio et al. 2006). Therefore co-operation

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between tDCs and mTECs leads to deletion of auto-reactive thymocytes and the establishment of central tolerance.

It seems clear that a TCR signal is essential for both positive and negative selection. However the mechanism by which cells interpret signals of varying strength to differentiate between survival and death remains unclear. A threshold for TCR signalling strength in order to discriminate between positive and negative selection has been described. The difference in affinity between the weakest ligand causing negative selection and the strongest ligand causing positive selection has been reported to be very close (Naeher et al. 2007). However the mechanism by which proximal signalling events are regulated to differentiate between strengths of TCR signalling is not well understood. It has been proposed that negative selection induces Erk phosphorylation at the cell membrane while positive selection induces Erk phosphorylation in the cytoplasm, possibly at the Golgi (Daniels et al. 2006). However more recently an alternative mechanism for differentiating TCR signals of varying strength in CD8<sup>+</sup> SP thymocytes has been proposed. This model states that in order for a self-antigen to induce negative selection it must co-ligate TCR and CD8 co-receptor for a minimum time. TCR and CD8 align through a zipper region on the TCR $\alpha$  and CD8 $\beta$  regions. This allows lck which is attached to the CD8 $\alpha$ , time to access the ITAMs located on the CD3 chains, allowing full  $\zeta$  chain phosphorylation. TCR antigen interactions which occur for less than the time needed for the TCR/co-receptor zipper action results in positive selection via the mechanisms described above, while complete phosphorylation of the  $\zeta$  chain



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results in negative selection (Palmer & Naeher 2009). It is thought that complete phosphorylation of the CD3  $\zeta$  chain during negative selection results in fully phosphorylated LAT leading to Grb2 and Sos1 recruitment in addition to SIp76, Gads, and PLC $\gamma$ 1 recruited during positive selection. This leads to strong transient activation of Erk along with p38 and JNK activation, as opposed to the weak prolonged expression observed during positive selection (Starr et al. 2003).

#### **1.4.2 Peripheral tolerance**

Negative selection of developing thymocytes based on their strong interaction with peptide/MHC complexes is fundamental to the establishment of central tolerance. A second form of tolerance known as peripheral tolerance is also essential for preventing auto-immunity. Peripheral tolerance is maintained by 2 principle mechanisms, T-cell anergy (passive) and the induction of T<sub>reg</sub> (active). T<sub>reg</sub> act to restrain pathogenic immune responses, stopping the outgrowth of potentially autoimmune T-cells which may have escaped negative selection. Although several subsets of cells have been implicated in restraining immune responses, FoxP3<sup>+</sup> T<sub>reg</sub> are the only currently known dedicated cell type which function in this way. Early investigations into the existence of T<sub>reg</sub> revealed the presence of a thymus derived cell type capable of mediating tolerance as observed in neonatal thymectomy experiments of chicken-quail chimeras (Ohki H et al. 1987). In studies involving the neonatal thymectomy of mice, it was

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observed that thymectomy between days 2 and 4 of birth resulted in autoimmunity and T-cell mediated lesions. Subsequent transfer of thymocytes from adult mice alleviated the observed pathology (Nishizuka Y 1969; B. Y. S. Sakaguchi, Takahashi & Nishizuka 1982a; Life 1996a). Following this it was found that a subset of CD4<sup>+</sup> SP cells with high surface expression of the  $\alpha$ -subunit of the IL-2 receptor (CD25) were capable of suppressing immune responses (S. Sakaguchi et al. 1995). Further understanding of the T<sub>reg</sub> lineage came from investigations into the human autoimmune disorder IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome). It was discovered that mutations in the X-chromosome encoding the FoxP3 gene were responsible for IPEX, as well as the corresponding mouse mutant scurfy (Chatila et al. 2000; Brunkow et al. 2001; Wildin et al. 2001). It was subsequently revealed in later studies that stable FoxP3 expression was restricted to T<sub>reg</sub> where it was essential for T<sub>reg</sub> function and differentiation (Fontenot et al. 2003)(Hori 2013). Loss of FoxP3 expression by T<sub>reg</sub> leads to a loss of suppressor function and the gain of effector T-cell properties (Williams & A. Y. Rudensky 2007).

### **1.4.3 Mechanisms of Treg immune suppression**

Several sophisticated mechanisms are used in order to prevent autoimmunity, maintain immune homeostasis and moderate inflammation. One of the principle cell types involved in maintenance of peripheral tolerance are T<sub>reg</sub>. Much work has gone into understanding the mechanisms by which T<sub>reg</sub> suppress immune responses. This has allowed insight into the control processes involved in

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tolerance but also offered potential therapeutic targets. In order to moderate immune responses  $T_{reg}$  can release inhibitory cytokines such as IL-10 and TGF $\beta$ . IL-10 and TGF $\beta$  are themselves suppressive cytokines, however they are also able to induce peripheral  $T_{reg}$  development. n $T_{reg}$  are thought to function primarily in a contact dependant manner, it therefore remains contentious as to the exact role IL-10 and TGF $\beta$  play in n $T_{reg}$  mediated suppression (Thornton & Shevach 1998; Shimon Sakaguchi 1998). There have however been reports in allergy models suggesting that both n $T_{reg}$  and induced antigen specific  $T_{reg}$  control disease, which is part dependent on IL-10 (Reviewed in Hawrylowicz et al. 2005). It therefore remains to be determined the exact role IL-10 and TGF $\beta$  play in n $T_{reg}$  immune suppression. A further mechanism for suppression is by cytotoxicity. Cytotoxic activity is mainly described in NK cells and CTLs (discussed in more detail in section 1.3.1). However naturally occurring human  $T_{reg}$  cells have also been shown to express granzyme A. Human  $T_{reg}$  have been shown to induce target cell death through perforin and granzyme A activity, via CD18 adhesion (Grossman et al. 2013).

Suppression by  $T_{reg}$  has also been shown to occur by a group of mechanisms which when broadly grouped together cause 'metabolic disruption' of target effector T-cells. One of these mechanisms, remains a source of considerable debate. As  $T_{reg}$  express high levels of CD25 it is proposed that  $T_{reg}$  deplete local IL-2, depriving effector T-cells of IL-2 mediated survival signals (Thornton & Shevach 1998). This mechanism has recently been proposed to be essential in  $T_{reg}$  mediated control of NK cells (Gasteiger et al. 2013). Prior studies have

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however disputed this as a mechanism for T<sub>reg</sub> mediated suppression (Fontenot, 2005)(Duthoit et al. 2005). Further suppression by T<sub>reg</sub> can occur via the intracellular or extracellular release of adenosine nucleosides by ectoenzymes. CD39 and CD73 have been shown to generate pericellular adenosine which can suppress effector T-cells by binding the Adenosine 2A receptor (A2AR) (Deaglio et al. 2007). Binding the A2AR both inhibits T-cell activation and stimulates TGFβ release. Release of TGFβ is then able to induce iT<sub>reg</sub> generation (Zarek et al. 2008). T<sub>reg</sub> have also been shown to target effector T-cells directly through transfer of the inhibitory secondary messenger cAMP. During cell-cell contacts with target cells, T<sub>reg</sub> are able to release cAMP through gap junctions (Bopp et al. 2007).

In addition to direct effects by T<sub>reg</sub> on effector T-cells, more recently it has become apparent that T<sub>reg</sub> are able to modulate APC function. In doing so T<sub>reg</sub> are able to reduce T-cell activation. T<sub>reg</sub> express high levels of cytotoxic T lymphocyte antigen 4 (CTLA-4). CTLA-4 is able to interact with CD80/86, a characteristic it shares with the stimulatory receptor CD28. CTLA-4-CD80/86 interaction was initially thought to supply an inhibitory signal to DCs, reducing their ability to activate T-cells (Reviewed in Rudd & Rudd 2009). However more recently it has become apparent that CTLA-4 can actually capture CD80/86 from the cell surface of DCs. These CTLA-4-CD80/86 complexes are then endocytosed by the CTLA-4 expressing cells and degraded (Qureshi et al. 2011). This results in impaired CD28 co-stimulation of effector T-cells and the dampening of the immune response. Some studies have also suggested a role

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for lymphocyte activation gene 3 (LAG3) in  $T_{reg}$  suppression of DCs. LAG3, expressed in the  $T_{reg}$  surface is thought to interact with MHC class II molecules with high affinity where it may block DC maturation (Huang et al. 2004). There have been many described mechanisms for  $T_{reg}$  immune suppression. It currently remains unclear which, if any, are the most crucial for  $T_{reg}$  to moderate immune responses.  $T_{reg}$  suppression seems relies on cell-cell contact, however the relative contribution by the numerous mechanisms remains the focus of on going research.

#### **1.4.4 FoxP3<sup>+</sup> Regulatory T-cells**

The X-chromosome encoded transcription factor FoxP3 is the lineage defining transcription factor for  $T_{reg}$ . FoxP3 is a member of the forkhead winged-helix family of transcription factors. Expression of FoxP3 is indispensable for the differentiation and function of  $T_{reg}$  cells (Williams & A. Y. Rudensky 2007). Mice with a T-cell specific deletion of FoxP3 lack  $T_{reg}$  and subsequently succumb to an overwhelming autoimmune pathology (Fontenot, Rasmussen, Williams, et al. 2005). The FoxP3 locus is comprised of 11 exons, the last of which remains untranscribed (Ying Wang et al. 2008). The FoxPs locus also contains 3 proximal conserved non-coding sequences (CNS1-3) within several intronic sequences. These are reported to play a prominent role in the regulation of stable FoxP3 expression. CNS3 contains a DNase I hypersensitive site and enriched with histones associated with promoter regions (H3K4me1). Nf- $\kappa$ B element c-Rel is reported to bind to CNS3 and regulate the FoxP3 promoter (Y. Zheng et al. 2010). CNS1 deficiency leads to impaired peripheral FoxP3 induction in

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peripheral T<sub>reg</sub> (A. Y. Rudensky 2011). CNS2 also known as the T<sub>reg</sub> specific demethylated region (TSDR) contains a CpG island the methylation status of which correlates with the stability of FoxP3 expression. The TSDR remains demethylated in ex-vivo FoxP3<sup>+</sup> T<sub>reg</sub> but fully methylated in conventional T-cells (Floess et al. 2007; Kim & Leonard 2007).

## **1.5 FoxP3<sup>+</sup> T<sub>reg</sub> compartment**

### **1.5.1 iTreg cells and their development**

FoxP3<sup>+</sup> T<sub>reg</sub> play a critical role in controlling auto-reactive immune responses. However the FoxP3<sup>+</sup> compartment contains more than one lineage of cell. While FoxP3 expression in the thymus leads to nT<sub>reg</sub> development, induction of FoxP3 in CD4<sup>+</sup> naïve T-cells in the periphery leads to generation of iT<sub>reg</sub>. The differentiation of nT<sub>reg</sub> is caused by increased self-reactivity of developing thymocytes. However iT<sub>reg</sub> generation most likely occurs upon interaction of naïve CD4<sup>+</sup> T-cells with allergens and commensal microbiota (Lathrop et al. 2011). iT<sub>reg</sub> are thought to develop in response to distinct TCR and ligand specificity, however their exact role in the mediation of immune tolerance still remains to be completely understood (Lathrop et al. 2008). It has been shown that TCR transgenic T-cells which begin to express FoxP3 after administration of cognate antigen are able to alleviate antigen induced airway inflammation (Mucida et al. 2005). iT<sub>reg</sub> may therefore function to control allergen induced inflammation particularly at mucosal membranes.

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As well as strong TCR signalling and suboptimal co-stimulation, generation of iTreg from naïve CD4<sup>+</sup> T-cells depends on TGFβ signalling (S. G. Zheng et al. 2004). TGFβ signalling leads to activation of the smad signalling pathway. The CNS1 region within the FoxP3 locus (discussed in section 1.3.4) contains both NFAT, smad3 and RARα binding sites. Analysis of CNS1 deficient mice shows defects in the generation of peripheral T<sub>reg</sub>. However CNS1 deletion does not result in detectable auto-immunity (Y. Zheng et al. 2010). This could suggest a non-redundant role for peripherally generated iT<sub>reg</sub> compared to thymically derived nT<sub>reg</sub> (Y. Zheng et al. 2010). FoxP3 induction after strong exposure to antigen and TGFβ is accompanied by decreased proliferation. This can be explained by the observation that TCR and TGFβ signalling stop recruitment of the cell cycle dependant methyl transferase (Dnmt 1) to the FoxP3 locus (most likely CNS2). This allows the FoxP3 locus to remain demethylated, stabilising expression (Josefowicz et al. 2012). Therefore sustained FoxP3 expression in iT<sub>reg</sub> relies on a combination of TCR and TGFβ signalling, as well as reduced cellular proliferation.

### **1.5.2 Thymically derived ‘natural’ T<sub>reg</sub>**

Naturally occurring regulatory T-cells (nT<sub>reg</sub>) are a subset of CD4<sup>+</sup> SP T-cell which develop in the thymus. They are characterised by high surface expression of the α-subunit of the IL-2 receptor CD25, as well expressing high levels of GITR and CTLA-4. They constitute approximately 5-10% of total CD4<sup>+</sup> SP T-cells in the periphery. nT<sub>reg</sub> function by maintaining self-tolerance and immune homeostasis by tempering immune responses to infectious agents

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(Reviewed in Josefowicz & A. Rudensky 2009). Strictly speaking  $nT_{reg}$  are not a subtype of effector T-cell as they are not formed from naïve T-cells in inflammatory conditions. However naïve T-cells can give rise to alternative forms of regulatory T-cells known as  $iT_{reg}$  and Tr1 cells.  $iT_{reg}$  develop in the presence of  $TGF\beta$  following TCR stimulation and produce large amounts of IL-10 and  $TGF\beta$ . Unlike most other  $CD4^+$  T-cell populations,  $iT_{reg}$  display immune suppressive activity with minimal antigen specificity (Reviewed in Weiner & Weiner 2001). Tr1 cells are an additional type of suppressive cell induced to form from naïve  $CD4^+$  SP T-cells. These cells however differ from  $nT_{reg}$  and  $iT_{reg}$  by lacking expression of the transcription factor FoxP3 (Vieira PL, 2004).

#### **1.5.3.1 TCR signal strength in $nTreg$ development**

During thymic T-cell development TCR avidity and duration instruct commitment to the  $CD4^+$  and  $CD8^+$  lineages (Reviewed in Singer et al. 2008). As well as  $CD4$  and  $CD8$  fate determination TCR signalling can instruct the development of specialised T-cell populations such as NKT cells and  $CD8\alpha\alpha$  T-cells (Leishman et al. 2002; Moran, Holzapfel, Xing, Cunningham, Maltzman, Punt & K. A. Hogquist 2011). It therefore comes as no surprise that TCR signalling also instructs the intra-thymic development of  $T_{reg}$ . TCR signals have been shown to be essential for thymic  $T_{reg}$  development. Studies show that thymic  $T_{reg}$  development only occurs in TCR transgenic mice following the introduction of a



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second transgene encoding the cognate ligand (Jordan et al. 2001). Previous to this it was shown that TCR transgenic mice specific for myelin basic protein, which cannot undergo endogenous rearrangement due to being RAG deficient, develop brain lesions. However in RAG sufficient hosts the inflammatory brain lesions are prevented by cells which possess endogenous TCRs (Yijie Wang & Lafaille 1998; Lafaille et al. 1994). Therefore TCR dependant signals are essential for T<sub>reg</sub> development and the establishment of tolerance. It is proposed that strong TCR signals, of a strength between that normally needed for positive and negative selection, cause FoxP3 expression and a T<sub>reg</sub> lineage commitment. Early evidence of this came from the observation that T<sub>reg</sub> express high levels of CD25, CD5 and CTLA-4, all of which are induced by TCR signalling. Further evidence for this came from retroviral transfer of T<sub>reg</sub> or naïve T-cell TCR $\alpha$  libraries into RAG<sup>-/-</sup> TCR transgenic T-cells. T<sub>reg</sub> derived TCRs were more able to induce expansion and auto-immunity upon transfer into lymphopenic hosts however only able to mount weak in vitro responses to syngenic APCs, when compared to the reaction to 'foreign' peptide recognised by their transgenic TCRs (C.-song Hsieh et al. 2004). Therefore the affinity range of conventional T-cells which recognise antigen during immune responses appears to be higher than that of T<sub>reg</sub> TCRs for self antigens. T<sub>reg</sub> development is likely to be instructed by TCR signals above that which normally cause positive selection of non-regulatory T-cell populations, but below that of negative selection. An instructive role for TCR signalling is also demonstrated by sequence analysis of mice bearing a single transgene encoded TCR $\beta$  chain. These mice show very little overlap between TCR $\alpha$  sequences of T<sub>reg</sub> and non-

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T<sub>reg</sub> (C.-song Hsieh et al. 2004). This suggests TCRs of distinct affinity for self ligands instruct development into either a conventional T-cell or a T<sub>reg</sub>. Studies using TCR transgenic mice which express a single T<sub>reg</sub> derived TCR suggest clonal competition between T<sub>reg</sub> for these high affinity self-ligands in the thymus. Normally T<sub>reg</sub> TCR transgenic mice contain very few T<sub>reg</sub>, however when precursor numbers are reduced, efficient T<sub>reg</sub> generation is observed. Suggesting that developing thymocytes compete for high affinity peptide ligands during development. In conditions of high clonal competition, such as in TCR transgenic mice this can lead to inefficient T<sub>reg</sub> induction (Bautista et al. 2009).

The studies discussed above suggest an important role for TCR signalling in the thymic selection of regulatory T-cells. However others have argued against this being the case. It was observed that in 3A9 and KRN TCR transgenic mice thymocytes underwent negative selection rather than differentiating into T<sub>reg</sub> when exposed to cognate antigen, encoded for by a second transgene in the thymus (Shih et al. 2004). The use of AND TCR transgenic mice further support this. Increasing the dose of TCR cognate ligand, cytochrome c, whose expression is under control of doxycycline, led to similar numbers of T<sub>reg</sub>. However T<sub>reg</sub> percentages were elevated owing to increased negative selection of conventional T-cells (Santen et al. 2004). A further study also suggested commitment to the T<sub>reg</sub> lineage occurs in the DN stage of development prior to full TCR rearrangement (D.Pennington, A.Hayday 2006). Therefore there was a period of time when the role which TCR signalling played in T<sub>reg</sub> selection

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remained unclear. However more recently a significant body of work seems to support a clear role for TCR specificity in thymic T<sub>reg</sub> selection.

#### **1.5.3.2 Role of co-stimulation in nT<sub>reg</sub> development**

In addition to TCR signals, CD28 co-stimulation appears to play a crucial cell intrinsic role in FoxP3 induction. Both CD28<sup>-/-</sup> and CD80/86<sup>-/-</sup> mice show severe reductions in T<sub>reg</sub> frequencies (Solomen et al. 2000)(Tai et al. 2005). Removal of the Ick binding domain present on the cytoplasmic tail of CD28 is also required for FoxP3 induction (Tai et al. 2005). This suggests that an important role for the co-operation of TCR and CD28 signalling in T<sub>reg</sub> selection. Further supporting this is the observation that many transcription factors usually associated with TCR and CD28 activation have been shown to contain binding sites on the FoxP3 locus. NFAT and AP1 bind to the FoxP3 promoter following increases in intracellular Ca<sup>2+</sup> after TCR and CD28 activation (Mantel et al. 2006). Various components of the TCR dependant NF-κB signalling pathway have also been shown to affect FoxP3 expression. Knockouts of CARMA1, PKCθ, Bcl10 and IKK2 have all been shown to reduce T<sub>reg</sub> frequencies (Reviewed in Josefowicz & A. Rudensky 2009). In addition to this Foxo1 and Foxo3 have also been shown to bind to the FoxP3 promoter (Ouyang, Beckett, Ma, Paik, et al. 2010). Previous studies have implicated a wide array of transcription factors associated TCR and CD28 signalling in FoxP3 induction. However the mechanism by which FoxP3 induction actually occurs remains incompletely understood.

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### 1.5.3.3 Cytokine signals in nTreg development

TCR sequence appears crucial for instructing commitment to the T<sub>reg</sub> lineage, however TCR signalling alone is insufficient for efficient T<sub>reg</sub> development. Only small percentages of thymocytes in TCR transgenic mice on a RAG deficient background differentiate into T<sub>reg</sub>, the rest become anergic T-cells (Apostolou et al. 2002). Also sequence analysis of TCR repertoires expressed on T<sub>reg</sub> and conventional T-cells show a partial overlap, suggesting TCR sequence alone is insufficient to ensure a commitment to the T<sub>reg</sub> lineage (C.-song Hsieh et al. 2004). T<sub>reg</sub> generation has been shown to be delayed in neonatal mice compared to conventional T-cell populations (Fontenot et al. 2005). This is despite a relative enrichment of auto-reactive T-cells in neonates due to lack of terminal deoxynucleotidyl transferase expression. In addition neonates contain high frequencies of CD25<sup>Hi</sup> FoxP3<sup>-</sup> T<sub>reg</sub> precursors (Chan-Wang Joaquim Lio & C.-S. Hsieh 2008). Delayed T<sub>reg</sub> differentiation has therefore been suggested to be due to the lack of a cytokine signal.

The primary cytokine thought to play a role in thymic T<sub>reg</sub> differentiation is IL-2. Mice deficient in the high affinity  $\alpha$ -subunit of the IL-2 receptor (CD25) or the IL-2R $\beta$  (CD122) exhibit lethal autoimmunity, displaying an approximate 50% reduction in T<sub>reg</sub> frequencies (Sadlack et al. 1993)(Suzuki H et al. 1995). The IL-2 receptor is also comprised of a third subunit, the common gamma chain ( $\gamma$ c). The other  $\gamma$ c cytokines IL-15 and IL-7 have also been described to play a role in thymic T<sub>reg</sub> differentiation. The IL-15R structure is related to that of the IL-2R, sharing both CD122 and  $\gamma$ c components. The IL-15R $\alpha$  chain in mTEC trans

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presents IL-15 to developing thymocytes (Stonier & Schluns 2010). Whereas IL-2<sup>-/-</sup> mice show a 50% reduction in T<sub>reg</sub> frequencies, IL-15<sup>-/-</sup> and IL-7<sup>-/-</sup> show no defects in the size of the T<sub>reg</sub> compartment (Vang et al. 2008). However  $\gamma$ c<sup>-/-</sup> mice contain no T<sub>reg</sub> whatsoever (Vang et al. 2008). Although T<sub>reg</sub> differentiation appears inhibited in IL-2<sup>-/-</sup> mice, T<sub>reg</sub> are not completely absent. This suggests some degree of overlap in the roles  $\gamma$ c cytokines play in T<sub>reg</sub> differentiation. Studies have shown that young IL-2<sup>-/-</sup> and IL-2R $\alpha$ <sup>-/-</sup> mice contain normal CD4<sup>+</sup> FoxP3<sup>+</sup> T<sub>reg</sub> frequencies. In contrast IL-2R $\beta$ <sup>-/-</sup> and IL-15<sup>-/-</sup> IL-2<sup>-/-</sup> double ko mice contain severely reduced CD4<sup>+</sup> FoxP3<sup>+</sup> T<sub>reg</sub> frequencies (Burchill et al. 2007). Perhaps suggesting a redundant role for IL-2 and IL-15 in T<sub>reg</sub> differentiation. IL-7 may also play a role, although most studies report no affect on T<sub>reg</sub> frequencies in IL-7<sup>-/-</sup> mice, unpublished data from Bayer and Malek suggest reduced T<sub>reg</sub> frequencies in IL-7R<sup>-/-</sup> mice (Malek 2008 unpublished data). Also thymic-stromal derived lymphopoietin which is itself implicated in human T<sub>reg</sub> development utilizes the IL-7R $\alpha$  chain. It therefore remains possible that IL-7R signaling in tandem with IL-2 act as the  $\gamma$ c cytokines regulating T<sub>reg</sub> differentiation (Hanabuchi et al. 2010).

Developing CD4<sup>+</sup> FoxP3<sup>+</sup> T<sub>reg</sub> express IL-2R $\beta$ , IL-7R $\alpha$  and IL-15R $\alpha$  and have been shown to respond to IL-7 and IL-2 and to a much lesser extent IL-15 (Vang et al. 2008). The molecular mechanisms of  $\gamma$ c cytokine signalling involved in T<sub>reg</sub> cell development remain incompletely understood. There appears to be an important role for STAT5 signalling as this is activated downstream of  $\gamma$ c cytokine signalling and contains binding sites on both the FoxP3 promoter and

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CNS2 region (M. a Burchill et al. 2008). Although IL-2-STAT5 signalling appears to play an essential role in T<sub>reg</sub> development it remains unclear whether STAT5 directly drives FoxP3 expression, induces changes in chromatin structure at the FoxP3 locus or promotes survival and proliferation of FoxP3<sup>+</sup> T<sub>reg</sub>. Deletion of a conditional STAT5 allele at the DP stage of development results in a dramatic decrease in FoxP3<sup>+</sup> T<sub>reg</sub> frequencies (M. A. Burchill et al. 2007). However there is some suggestion that introduction of a Bcl-2 transgene into STAT5 deficient cells results in the rescue of STAT5 deficient T<sub>reg</sub> (Josefowicz & A. Rudensky 2009- personal communication with S.Malin and M. Busslinger). This firstly shows STAT5 independent T<sub>reg</sub> differentiation and secondly suggests a role for STAT5 signalling in regulating survival and proliferation.

In addition to its role in FoxP3 induction in thymic T<sub>reg</sub> development, IL-2 is suggested to play a role in T<sub>reg</sub> homeostasis. IL-2<sup>-/-</sup> and IL-2R<sup>-/-</sup> mice contain ten-fold reductions in CD4<sup>+</sup> FoxP3<sup>+</sup> T<sub>reg</sub> in the periphery. These cells express low levels of FoxP3 and CD25, suggesting IL-2 plays an important role in enhancing FoxP3 expression (Fontenot, Rasmussen, Gavin & A. Y. Rudensky 2005)(Bayer et al. 2007)(Dennis Adeegbe,\* Allison L. Bayer,\*† Robert B. Levy n.d.). Gene expression profiling of T<sub>reg</sub> from IL-2<sup>-/-</sup> mice before and after IL-2 treatment showed up regulation of mRNAs related to growth as opposed to immune function (Fontenot, Rasmussen, Gavin & A. Y. Rudensky 2005). In addition to this BrdU uptake was lower in peripheral T<sub>reg</sub> from mice with impaired expression of IL-2R (Malek et al. 2002)(Bayer et al. 2007). Peripheral T<sub>reg</sub> production is also dependant on IL-2 as adoptively transferred CD4<sup>+</sup> T-cells

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from IL-2<sup>-/-</sup> mice but not IL-2Rα<sup>-/-</sup> mice protect IL-2 sufficient hosts from EAE, leading to an increase in donor CD4<sup>+</sup> CD25<sup>+</sup> T-cells, that were dependant on host derived IL-2 (Furtado et al. 2002). Thus IL-2 in the periphery seems to play an important role in T<sub>reg</sub> growth and proliferation, as well as enhancing FoxP3 expression.

Transforming growth factor β (TGFβ) is a regulatory cytokine with pleiotropic functions in control of the immune system. The TGFβ-Smad pathway plays a pivotal role in development, maintenance, survival and function of T-cells (M. O. Li & R. A. Flavell 2008). There are 3 isoforms of TGFβ in mammals, TGFβ1, TGFβ2 and TGFβ3. These signal through two trans-membrane serine-threonine kinase receptors, TGFβR1 and TGFβR2. TGFβ1 is expressed predominantly during regulation of the immune system (Govinden & Bhoola 2003). Mice with a T-cell specific deletion of TGFβ1 or TGFβR1 develop early fatal autoimmunity, highlighting an important role for TGFβ in regulating immune tolerance (Shull et al. 1992)(Marie et al. 2005). T-cells appear to be the main cause of the autoimmunity associated with TGFβ ablation as CD4<sup>+</sup> or CD8<sup>+</sup> depletion alleviates the inflammatory phenotype (Letterio et al. 1996). However, as TGFβ1 is able to modulate multiple cell types it was previously unclear whether TGFβ1 was directly or indirectly responsible for the observed inflammation (M. O. Li & R. A. Flavell 2008). Studies attempted to address this using a dominant negative TGFβRII on either a CD4 or CD2 promoter (DNTGFβR)(Gorelik & R. A. Flavell 2000)(Lucas et al. 2000). These mice, unlike TGFβ<sup>-/-</sup> mice, survive to adulthood but still develop an inflammatory disease. More recent studies

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suggest this is due to incomplete ablation of TGF $\beta$  signalling in DNTGF $\beta$ R mice as mice contain nuclear phospho-SMAD as a result of TGF $\beta$  signalling. Other studies attempted to study the role of TGF $\beta$  in nT<sub>reg</sub> development through the use of conditional knock outs of the TGF $\beta$ R components (M. O. Li et al. 2006)(Marie et al. 2006)(Ouyang, Beckett, Ma & M. O. Li 2010). These also report severe inflammation which is attributable to increased T-cell activation, but fail to come to any consensus on T<sub>reg</sub> cell dysfunction. However recently a more direct approach on the role TGF $\beta$  plays in nT<sub>reg</sub> development has been published by Harold von Boehmer's group. In this study mice are generated which lack the Smad binding site exclusively on the FoxP3 CNS1 region (FoxP3<sup>CNS1mut</sup>). They show that binding of Smad3 to the FoxP3 CNS1 enhancer is dispensable for thymic T<sub>reg</sub> cell development, and only required for development within the gut (Schlenner et al. 2012).

#### **1.5.4 Plasticity**

FoxP3 expression plays a central role in maintaining the T<sub>reg</sub> transcriptional program. Therefore continued FoxP3 expression is critical to the stability of the T<sub>reg</sub> lineage and continued maintenance of peripheral homeostasis. As well as stimulating transcription of various genes, FoxP3 acts to repress the expression of others, such as IL-2, TNF- $\alpha$ , IFN- $\gamma$ , IL-17 and IL-4 (Gavin et al. 2007)(Wan & R. A. Flavell 2007). Repression of pro-inflammatory cytokine secretion is of particular importance in T<sub>reg</sub> as they express TCRs with increased affinity for self peptide. T<sub>reg</sub> therefore possesses the ability to potentially mount autoimmune responses in the absence of FoxP3 expression (Apostolou et al. 2002).



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(Apostolou et al. 2002) (C.-song Hsieh et al. 2004). Illustrating this, in FoxP3 deficient mice, activated T-cells express TCRs which would normally be present on T<sub>reg</sub> in FoxP3 sufficient animals (C.-song Hsieh et al. 2006). Suggesting self-reactive T-cells developed into auto-reactive conventional T-cells rather than being diverted in the T<sub>reg</sub> lineage. This would suggest that the stability of FoxP3 expression in T<sub>reg</sub> is of central importance in order to maintain immune homeostasis, and prevent ex-T<sub>reg</sub> mediated autoimmunity.

In order to investigate the stability of FoxP3 expression in vivo the Bluestone group generated FoxP3-GFP Cre R26 mice (Zhou et al. 2009). This allowed the identification of a substantial population of YFP<sup>+</sup> GFP<sup>-</sup> 'exT<sub>reg</sub>' cells. These cells were reported to have transiently up regulated FoxP3 expression before gaining an activated memory T-cell phenotype, producing inflammatory cytokines. This study however fails to investigate the possibility that committed T<sub>reg</sub> cells stably express FoxP3 and represent a stable committed cell lineage. To further investigate this FoxP3<sup>GFP-Cre-ERT2</sup> R26 mice were generated by the Rudensky lab. In these mice the Cre-ERT2 fusion protein is sequestered in the cytosol, therefore YFP is not expressed. Subsequent treatment with tamoxifen allows for nuclear translocation of the fusion protein, excision of the R26 STOP cassette and YFP expression. This therefore allows the analysis of inheritable YFP expression in cells which expressed FoxP3 at the time. This study observed remarkably stable T<sub>reg</sub> FoxP3 expression, in both basal conditions and radiation-induced lymphopenia as well as in Th1 cytokine induced inflammatory responses (Rubtsov et al. 2010). Therefore T<sub>reg</sub> seem to represent a stable

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lineage with dedicated mechanisms for maintaining immune homeostasis and peripheral tolerance.

### **1.5.5 Models for nTreg development**

Since the discovery that the thymic expression of FoxP3 regulates development of T<sub>reg</sub> much work has focussed on determining the signal cues which lead to induction of FoxP3 expression. As discussed above, both TCR and cytokine signalling appear to play crucial roles in ensuring thymocytes commit to the T<sub>reg</sub> lineage. However it still remains unclear how these signals co-operate to ensure efficient T<sub>reg</sub> differentiation. Current data appears to support self-reactivity as the primary determinant in committing to the T<sub>reg</sub> lineage. However some previous studies have hinted at a TCR-independent signal during the DN stage of development playing a crucial role. Hayday and colleagues show that the propensity of early TCR $\alpha\beta$ <sup>+</sup> CD4<sup>-</sup> CD8<sup>-</sup> DN thymocytes to differentiate into FoxP3<sup>+</sup> T<sub>reg</sub> is regulated in trans by CD4<sup>+</sup> CD8<sup>+</sup> DP thymocytes (Daniel J. Pennington, 2006). This suggests a stochastic model for development in which T<sub>reg</sub> express FoxP3 prior to TCR signalling. In this model thymocytes which express FoxP3 and undergo strong selection signals become T<sub>reg</sub>. While FoxP3<sup>-</sup> cells which have increased self-reactivity undergo negative selection.

More recent models however describe an instructive role for TCR signalling in FoxP3 expression (evidence for/against discussed in section (1.5.2.1). In these models increased self-reactivity of developing thymocytes supports FoxP3

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expression and T<sub>reg</sub> development. Renewed support for this model came from studies using TCR transgenic mouse lines which express TCRs derived from nT<sub>regs</sub>. Contrary to what was expected, these mice contained virtually no T<sub>reg</sub> (C.-S. Hsieh et al. 2012). However after making bone marrow chimeras, it was found that reducing precursor frequency supported efficient T<sub>reg</sub> development. The authors suggest the experimental artefact of a thymus in which all thymocytes express identical TCR led to intra-clonal competition for identical peptide ligands. After increasing clonal frequencies T<sub>reg</sub> percentages were observed to plateau lower than that of conventional CD4<sup>+</sup> SPs (Bautista et al. 2009). This suggested a model for T<sub>reg</sub> development in which competition for a single interaction with a rare peptide ligand caused T<sub>reg</sub> development. This peptide ligand is hypothesised to be rarer than that which causes positive selection of conventional T-cells, as T<sub>reg</sub> percentages remain lower than that of FoxP3<sup>-</sup> CD4<sup>+</sup> SPs at increasing clonal frequencies. T<sub>reg</sub> frequencies failed to drop at higher clonal frequencies suggesting one single competitive event for a high affinity peptide occurs during T<sub>reg</sub> differentiation, rather than multiple competitive events.

Rare high affinity TCR/self-peptide interactions are consistent with the previously described 'hit and run' model for FoxP3 induction during thymic development (Ouyang & M. O. Li 2011). This model suggests that Nf-κB and Foxo activation are oppositely regulated by TCR and CD28 stimulation to turn on FoxP3 gene transcription. In the presence of high affinity antigen stimulation TCR and CD28 signals activate Nf-κB via IKK, while inactivating Foxo proteins

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via Akt. Foxo proteins have been shown to co-operate with FoxP3 to control the differentiation of T<sub>reg</sub> (Ouyang, 2010). Therefore multiple high affinity signalling events are not conducive to FoxP3 expression and T<sub>reg</sub> development. However upon cessation of high affinity antigen stimulation, Foxo proteins can relocate back to the nucleus where it can co-operate with Nf-κB to induce FoxP3 gene transcription. This model would therefore predict that frequent high affinity antigen stimulation would lead to apoptosis and negative selection, while sparse high affinity signalling events lead to FoxP3 expression and T<sub>reg</sub> development. The 'hit and run' model however fails to include a direct role for IL-2 mediated STAT5 signalling in causing FoxP3 induction. It remains possible that IL-2 co-operates with Foxo and FoxP3 in causing T<sub>reg</sub> differentiation, while it may also play a homeostatic role perhaps enhancing FoxP3 transcription and T<sub>reg</sub> cell survival.

Following the described role for IL-2 in T<sub>reg</sub> differentiation as well as the characterisation of CD25<sup>Hi</sup> FoxP3<sup>-</sup> precursors of T<sub>reg</sub> cells in neonatal mice, Lio and Hsieh proposed a 2 step model for T<sub>reg</sub> differentiation (Chan-Wang Joaquim Lio & C.-S. Hsieh 2008). This 2 step model proposes a TCR dependant phase of development in which a high avidity TCR signal results in CD25 up-regulation. This is followed by a cytokine dependant phase of development in which an IL-2 signal causes FoxP3 expression. This is likely to occur via STAT5, as STAT5 binding domains are located on both the FoxP3 locus as well as the CNS2 regulatory element (M. Burchill et al. 2008) (discussed in section 1.5.2.2). The 2 step model therefore describes IL-2 as the main driving force in

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causing FoxP3 expression rather than TCR/CD28 co-stimulatory signalling. However IL-2<sup>-/-</sup> mice still contain approximately 50% of their T<sub>reg</sub> compartment (Furtado et al. 2002)(Malek et al. 2002). It therefore seems unlikely this is the whole story. The identity of the T<sub>reg</sub> precursors as well as the mechanisms by which they differentiate into T<sub>reg</sub> therefore still remain unclear. The observed delay in T<sub>reg</sub> differentiation compared to that of conventional non-regulatory populations also remains incompletely understood. Further analysis of this delay in development may elude crucial information as to the signalling pathways involved in precursor differentiation.

## **1.6 Aims**

Many previous studies have aimed to determine the signalling cues which lead to the thymic differentiation of nT<sub>reg</sub>. Many of these studies use adult mice in which the T-cell compartment is under homeostatic control. After thymic egress, studies have shown recirculation of considerable populations of FoxP3<sup>+</sup> cells back to the thymus (McCaughy et al. 2007). Therefore changes in the frequency of FoxP3<sup>+</sup> cells observed after alterations in signalling may in fact be due to changes in peripheral homeostasis. In addition no marker currently exists which is capable of distinguishing peripherally derived iT<sub>reg</sub> from nT<sub>reg</sub> which are of thymic origin. It therefore remains difficult to distinguish between factors which directly affect nT<sub>reg</sub> differentiation and those which may alter frequencies of peripheral T<sub>reg</sub> populations.

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Similar problems have been encountered when studying potential nT<sub>reg</sub> precursor populations. Adult mice which possess fully reconstituted immune systems contain thymic cellular compartments otherwise absent from neonatal mice. When studying precursor-product relationships in adults it can be difficult to determine between newly developed progenitor populations and thymic recirculants. This can be a particular problem when studying T<sub>reg</sub> as naïve T-cells are capable of FoxP3 expression when stimulated appropriately. Therefore in this study our aims include :-

1. The study of the timing of de novo T<sub>reg</sub> development relative to other T-cell populations.
2. Analysis of T<sub>reg</sub> precursor populations in the absence of recirculating cell types.
3. Characterisation of the signalling cues required for FoxP3 expression.
4. Identification of the thymic cell types responsible for the delivery of T<sub>reg</sub> differentiation signals.

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## Chapter 2

### Materials and Methods

**Table 2.1 Mice**

Strain	Reference
C57/Bl/6	/
Rag1 <sup>-/-</sup>	(Mombaerts et al. 1992)
FoxP3 <sup>GFP</sup>	(Ying Wang et al. 2008)
TetZap70	(Saini et al. 2010)
OTI	(K. A. Hogquist et al. 1994)
OTII	(Barnden et al. 1998)
IKK2 <sup>Fl/Fl</sup> CD4 Cre R26	(Z.-wei Li et al. 2013; Srinivas et al. 2001)
IL-2 <sup>-/-</sup> Rag1 <sup>-/-</sup>	(Schorle et al. 1991)
IL-15Rα <sup>-/-</sup> Rag1 <sup>-/-</sup>	(Kennedy et al. 2000)

#### Mice

The mice detailed above were bred and housed at NIMR animal facility under SPF conditions, in accordance with home office regulations. Genotyping was performed by a combination of flow cytometric analysis on peripheral blood lymphocytes and PCR using mouse tail DNA. All animals were maintained on normal dry feed with the exception of TetZap70 FoxP3<sup>GFP</sup> mice which when indicated were fed 3mg dox from birth. All mice were between 16-20 weeks of age when culled.

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## **Tetracycline inducible Zap70 mice**

Mice were generated bearing an inducible tetracycline transgene of Zap70 (Zap70<sup>TRE</sup>) and a tailless human CD2 (HuCD2) reporter construct. Expression was then be targeted within the T-cell lineage by breeding with mice which constitutively express the reverse tetracycline transactivator (rtTA) under the control of a human CD2 (HuCD2) expression element. Mice were then additionally back crossed onto Zap70<sup>-/-</sup> mice ensuring that all Zap70 expression was due to the transgene. These mice were then crossed with mice expressing GFP at their endogenous FoxP3 locus (GFP<sup>FoxP3</sup>), Zap70<sup>TRE</sup> rtTA<sup>HuCD2</sup> Zap70<sup>-/-</sup> GFP<sup>FoxP3</sup> mice (TetZap70 GFP<sup>FoxP3</sup>).

## **Doxycycline induction of TetZap70 GFP<sup>FoxP3</sup> mice.**

TetZap70 GFP<sup>FoxP3</sup> mice were induced to express Zap70 by the administration of the tetracycline derivative doxycycline (dox) in food, at a concentration of 3mg/g (3% w/w).

## **Media.**

Phosphate buffered saline (PBS) was made in house at NIMR or purchased from GIBCO. Fluorescent activated cell sorting (FACs) buffer – PBS, supplemented with 0.5% v/v sodium azide (Sigma) and 0.5% w/v bovine serum album (BSA) (Sigma). Air buffered Iscove's modified Dulbecco's medium (IMDM) – made in house at NIMR. Handling media – Air buffered IMDM containing 1% (w/v) BSA. Ack lysis buffer – 150mM NH<sub>4</sub>Cl, 10mM KHCO<sub>3</sub>, 0.1mM Ethylenediaminetetraacetic acid (EDTA).



## Antibodies

**Table 2.2 Cell surface and intracellular antibodies used for flow cytometry**

Specificity	Manufacturer	Working concentration
CD45.1-FITC	A20 (eBioscience)	2.5µg/ml
CD5-FITC	53-7.3 (eBioscience)	1.25 µg/ml
HuCD2-P.E	RPA-2.10 (BD)	diluted 1:50
CD45.1-P.E	A20 (eBioscience)	2.5 µg/ml
HSA-P.E	M1/69 (eBioscience)	0.5µg/ml
B220-P.E	RA3-6B2 (eBioscience)	1 µg/ml
CD8α-PETR	5H10 (eBioscience)	1µg/ml
TCR-Pecy5	H57-597 (eBioscience)	1µg/ml
CD25-Pecy7	PC61.5 (eBioscience)	1.25µg/ml
CD4-450eFlur	L3T4 (eBioscience)	2.5µg/ml
CD5-APC	53-7.3 (eBioscience)	1.25µg/ml
TCR-APC	H57-597 (eBioscience)	1µg/ml
CD44-APC-EF780	IM7 (eBioscience)	0.5µg/ml
FoxP3-APC	FJK-16a (eBioscience)	4 µg/ml
GITR-PE	DTA-1 (eBioscience)	0.5µg/ml
CTLA-4	14D3 (eBioscience)	
CD62L-PE	MEL-14 (eBioscience)	0.5µg/ml
Zap70-PE	1E7.2 (eBioscience)	4µg/ml
Vα2-bio	B20.1 (eBioscience)	0.5µg/ml
sav-PO	Invitrogen	5µg/ml

**Table 2.3 Blocking antibodies used.**

Specificity	Clone	Manufacturer	Amount injected
IL-2	JES6-1A12	BioXcell	0.5mg/injection
	JES6-5H4	BioXcell	0.5mg/injection
	S4B6	BioXcell	0.5mg/injection
TGFβ	1D11.16.8	BioXcell	1mg/injection
CD40L	MR1	BioXcell	0.5mg/injection
CD80	16-10A1	BioXcell	0.5mg/injection
CD86	GL-1	BioXcell	0.5mg/injection

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### **Preparation of single cell suspensions.**

Cervical, auxiliary, brachial, mesenteric and inguinal lymph nodes (LNs), spleen and thymus were all dissected from mice. Lymphocytes were mashed in ice cold handling buffer via manual disintegration through 75µm nylon mesh. Cells were then washed 1-2 times (1200 rpm, 4 mins) and re-suspended in FACs buffer. Total lymphocytes counts were then taken using a Casy-1 cell counter (Schärfe System) according to the manufacturers instructions. Cells were kept on ice throughout.

### **Obtaining bloods.**

100µl of blood was obtained by nicking the tail vein of experimental mice while restrained with a scalpel blade. Bloods were gathered into microfuge tubes containing 100µl heparin (made in PBS according to manufacturers instructions). 2ml of Ack buffer was then added to lyse the red blood cells before vigorous vortexing for 3 minutes. Samples were then washed in cold FACs buffer.

### **Flow cytometry.**

Lymphocytes were incubated on ice with 100µl primary antibody stain per  $1-2 \times 10^6$  cells. Antibodies were made up to the appropriate working concentration indicated in table 1 and once added to the cells left for 1 hour. Cells were then washed 1-2 times in FACs buffer and filtered through 35µm pore cell strainers (BD Flacon). Flow cytometry was performed on the Cyan-ADP (Beckman Coulter) or BD LSR II. Data analysis was then performed using Flowjo software

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(v8.8.6 or v9.01) (Tree star). Intracellular staining was carried out following the surface staining. Cells were washed and then fixed/permeabilised using the ebioscience FoxP3 fix/perm kit according to the manufactures instructions. When carrying out intracellular staining for FoxP3 in Nur77<sup>GFP</sup> mice, to avoid leaking of GFP, cells were pre treated with 0.5% paraformaldehyde for 15 mins at room temperature. Following this cells were stained using the ebioscience FoxP3 kit as previously described.

### **Bone marrow isolation.**

Bone marrow was isolated from the tibiae and femora of TetZap70 GFP<sup>FoxP3</sup>/C57B6 CD45.1/OTI/OTII/IKK2 CD4<sup>Cre</sup> R26 mice. Epiphyses were removed and bone marrow was obtained by flushing handling media through the medullary canal with a 25-guage needle. The bone marrow was then filtered through a 35µm cell strainer and washed twice in handling media before being re-suspended in handling media at a concentration of 25x10<sup>6</sup> cells/ml.

### **Bone marrow/Thymus T-cell depletion.**

Bone marrow suspensions were treated with TCRβ-BIO at the working concentration (table 1) for 30 minutes at 4°C. Cells were then washed in FACs buffer and re-suspend in 4ml of IMDM. 80x10<sup>6</sup> dyna beads were then added

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and left to rotate for 20 minutes at 4°C. The tube was then placed in the dyna magnet for 30 seconds and the supernatant removed, before repeating this step. The cells were then washed and re-suspended in IMDM and counted. The above was replicated for CD8<sup>+</sup> thymocytes depletion using CD8-bio, prior to 3-way sort on FACs Aria II or XDP.

### **Irradiation and bone marrow reconstitution.**

Rag1<sup>-/-</sup> host mice were irradiated with 500 rads (caesium source) and allowed to rest for 12-24 hours and subsequently treated with 0.02% (v/v) Baytril® for one month. Transfer of bone marrow cells was performed by intravenous injection of 5x10<sup>6</sup> bone marrow cells into the lateral tail vein or irradiated hosts with a 0.5ml microfine insulin syringe (BD). Mice were then left for 6 weeks to allow full reconstitution of the lymphoid compartment, which was then confirmed by phenotypic analysis of peripheral blood, looking for the presence of B-cells by staining for B220 and CD19 and analysing on the FACs calibur.

### **Intraperitoneal methacycline injections**

10mg/ml Methacycline (Sigma-Aldrich) solution, dissolved in PBS was neutralised using 1M NaOH. Irradiated mice then received 200µl of a 10mg/ml methacycline solution (2mg) in a single intraperitoneal injection using a 0.5ml microfine insulin syringe (BD).

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### **Blocking antibody treatment**

200µl of 10mg/ml TGFβ blocking antibody (1mg) (1D11.16.8, BioXcell) was injected into irradiated mice via a single intraperitoneal (ip) injection using a 0.5ml microfine insulin syringe (BD). This treatment was repeated for CD40L (MR1, BioXcell) blocking treatment. During IL-2 antibody blockade mice received 0.5mg JES6-5H4 and 0.5mg S4B6 antibody clones (BioXcell) in a single 400µl ip injection. CD80/CD86 blocking antibody injections contained 0.5mg of the CD80 neutralising antibody (16-10A1, BioXcell) and 0.5mg of the CD86 neutralising antibody (GL-1, BioXcell). All Blocking antibody treatments were begun 1 day prior to feeding mice dox and repeated every 2 days until culling.

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## Chapter 3

# Characterisation of de novo Thymic Regulatory T-cell development

### Introduction

Regulatory T-cells ( $T_{reg}$ ) are an essential subset of T-cells responsible for maintaining peripheral tolerance and immune homeostasis by applying a dominant inhibitory effect on lymphocytes (Seddon & Mason 2000)(Itoh et al. 1999).  $T_{reg}$  are  $\alpha\beta$  T cell Receptor (TCR) expressing T cells generated in the thymus during positive selection, and comprise approximately 5-10% total  $CD4^+$  SPs (Single positives).  $T_{reg}$  are classically defined by their constitutive expression of CD25 (high affinity  $\alpha$ -subunit of the IL-2 receptor), CTLA-4 and GITR. While these proteins may play a functional role in  $T_{reg}$ , none of them are specific to  $T_{reg}$  as activated T cells can also up-regulate expression. A forkhead family transcription factor FoxP3 has been shown to be a critical regulator of this lineage (Hori et al. 2003) (Fontenot et al. 2003) (Khatttri et al. 2003) and is to date the most reliable marker for thymus derived  $T_{reg}$ .

Although it is commonly accepted that  $T_{reg}$  develop in the thymus, the mechanism by which this occurs remains controversial, however cytokine signaling is thought to play a key role. IL-2 has been shown to be particularly important, since IL-2R $\beta^{-/-}$  mice show a decreased number of thymic  $T_{reg}$  (Malek 66

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et al. 2002). However the observation that IL-2<sup>-/-</sup> mice still contain approximately 50% of their T<sub>reg</sub> compartment suggests a non-essential role for thymic T<sub>reg</sub> induction (Fontenot, Rasmussen, Gavin & A. Y. Rudensky 2005).

Induction of FoxP3 and commitment to the T<sub>reg</sub> lineage also requires signalling through the TCR. In studies using monoclonal TCR transgenic mice, T<sub>reg</sub> will only develop in the presence of a second transgene encoding the thymic expression of cognate ligand (Jordan et al. 2001). The ability of the TCR to engage self-ligands with a specific affinity during development instructs commitment into a variety of T-cell lineages, including that of T<sub>reg</sub> cells. T<sub>reg</sub> appear to be selected on strong self-peptide interactions, with a TCR signalling strength between that needed for positive and negative selection. The increased TCR signal strength relative to non-regulatory T-cell populations is evident in mice expressing GFP from the immediate early gene Nr4a1 (Nur77) locus (Nur77<sup>GFP</sup>). In these mice GFP is up regulated upon antigen stimulation but not upon inflammatory signalling. GFP expression levels correlate with the strength of the TCR signal received (Moran, Holzapfel, Xing, Cunningham, Maltzman, Punt & K. a Hogquist 2011). In Nur77<sup>GFP</sup> mice T<sub>reg</sub> have higher GFP expression, suggesting thymic T<sub>reg</sub> receive strong selection signals during development.

Signalling through the TCR is mediated by various components associated with the TCR signalling complex. One of the key proximal kinases is ζ- Chain Associated Protein Kinase of 70 KDa (ZAP-70). Zap70 is essential for thymic development beyond the CD4<sup>+</sup> CD8<sup>+</sup> double positive (DP) stage. It is the

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signalling cascades initiated by TCR signalling and subsequent activation of intracellular kinases such as Zap70 which is thought to lead to FoxP3 expression and the commitment to the Treg lineage. However the location, the timing and indeed the type of ligand needed to instigate the signalling cascade is highly debated. A two step model for T<sub>reg</sub> development has been proposed by Lio and Hsieh, this combines both the requirement for TCR signaling with the requirement for IL-2. In this model it is proposed that strong TCR-self peptide interactions on developing thymocytes cause CD25 up regulation. This CD25 high 'precursor' population are then more responsive to paracrine IL-2 which can cause FoxP3 expression via activation of STAT5, instructing commitment to the T<sub>reg</sub> lineage.



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## Results

### Normal CD4<sup>+</sup> SP FoxP3<sup>+</sup> T<sub>reg</sub> development in inducible Zap70 mice

In order to examine de novo thymic T<sub>reg</sub> development in the adult thymus, we took advantage of Zap70<sup>-/-</sup> mice in which Zap70 expression is controlled by a tetracycline inducible Zap70 transgene (*TetZap70*) (Saini et al. 2010). Feeding TetZap70 mice the tetracycline derivative doxycycline restores Zap70 expression and therefore TCR signalling and positive selection. This alleviates the block at the CD4<sup>+</sup> CD8<sup>+</sup> DP (double positive) stage T-cell development in Zap70<sup>-/-</sup> mice. The TetZap70 mice also contain a tailless HuCD2 reporter allowing easy identification of thymocytes expressing the TetZap70 transgene. We wished to determine if thymic development of CD4<sup>+</sup> SP FoxP3<sup>+</sup> T<sub>reg</sub> was normal in TetZap70 mice. Therefore TetZap70 mice were fed doxycycline from birth and their thymus and lymph nodes analysed for the presence of CD4<sup>+</sup> SP FoxP3<sup>+</sup> T<sub>reg</sub>. At 16 weeks both the thymus and LNs of the TetZap70 mice contained FoxP3<sup>+</sup> T<sub>reg</sub> (Fig 1.1A). The absolute numbers of CD4<sup>+</sup> SP FoxP3<sup>+</sup> T<sub>reg</sub> in TetZap70 mice were similar to the WT thymus. However the numbers normally observed in the LN were higher when compared to the WT (Fig 1.1B). Therefore constitutive Zap70 expression in *TetZap70* mice is sufficient to generate FoxP3<sup>+</sup> CD25<sup>Hi</sup> T<sub>reg</sub>.

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### **CD4<sup>+</sup> SP FoxP3<sup>+</sup> T<sub>reg</sub> are detectable on d4 in TetZap70 FoxP3<sup>GFP</sup> mice**

Next we wanted to investigate more closely the timing and phenotype of the first wave of T<sub>reg</sub> to develop in our TetZap70 mice. To facilitate this we crossed TetZap70 mice with mice expressing EGFP from the endogenous FoxP3 locus (TetZap70 FoxP3<sup>GFP</sup>) (Ying Wang et al. 2008). These mice contain an IRES-EGFP allowing identification of FoxP3 expressing cells, while avoiding the use of a fusion protein which has been reported to affect the assembly of the FoxP3 signalling complex (Bettini et al. 2012). This allows us to identify when FoxP3 expressing cells develop during thymic selection and how induction of FoxP3 occurs. TetZap70 FoxP3<sup>GFP</sup> mice were fed dox and their thymus analysed at different timepoints for the presence of GFP<sup>+</sup> FoxP3 expressing cells by FACs. After day 2 we observed restoration of the CD4<sup>+</sup> SP compartment (Fig 1.2A) while CD8<sup>+</sup> SP development was delayed until approximately day 4. Looking within the CD4<sup>+</sup> SP compartment at the TCR<sup>Hi</sup> CD5<sup>Hi</sup> mature T-cells, the first GFP expressing cells were detected at day 4, with percentages steadily increasing until day 8 (Fig 1.2B). Analysing the CD4<sup>+</sup> CD8<sup>+</sup> DP compartment revealed a bimodal distribution of HuCD2 reporter expression ranging from approximately 10 to 80% of total DPs (Fig 1.2C). Therefore the TetZap70 FoxP3<sup>GFP</sup> mice had a large degree of variability in their mouse to mouse Zap70 transgene expression.

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### Thymic development in TetZap70 FoxP3<sup>GFP</sup> chimeras

In an attempt to get more consistent mouse to mouse transgene expression we analysed development of TetZap70 FoxP3<sup>GFP</sup> cells in bone marrow chimeras. *Rag1*<sup>-/-</sup> mice were sub lethally irradiated (500 rads) and injected with bone marrow from TetZap70 FoxP3<sup>GFP</sup> mice. Mice were left for six weeks to reconstitute, then fed dox and their thymus analysed by FACs. Consistent with the intact TetZap70 FoxP3<sup>GFP</sup> mice, CD4<sup>+</sup> SPs were first detected on day 2, while CD8<sup>+</sup> SP development was delayed until day 4 (Fig 1.3A). Analysing HuCD2 expression revealed more consistent transgene induction as reported by HuCD2 expression levels in the chimeras (Fig 1.3B). Interestingly percentages of GFP<sup>+</sup> T<sub>reg</sub> within the chimeras and intact TetZap70 FoxP3<sup>GFP</sup> mice appear similar until day 5. At which point percentages continued to steadily increase in the chimeras plateauing at a higher level than in the intact mice (Fig 1.3C). These results suggest that reconstituted TetZap70 FoxP3<sup>GFP</sup> chimeras express the Zap70 transgene more consistently, and appear able to generate increased percentages of T<sub>reg</sub> compared to intact TetZap70 FoxP3<sup>GFP</sup> mice. We next wished to determine if the observed differences in T<sub>reg</sub> thymic development were due to irradiation of the *Rag1*<sup>-/-</sup> hosts, or intrinsic to TetZap70 FoxP3<sup>GFP</sup> mice. To do this we made TetZap70 FoxP3<sup>GFP</sup> chimeras using either TetZap70 FoxP3<sup>GFP</sup> or *Rag1*<sup>-/-</sup> mice as hosts. After 6 weeks mice were put on dox and their thymus analysed by FACs. CD4<sup>+</sup> SP GFP<sup>+</sup> T<sub>reg</sub> were detectable in the TetZap70 FoxP3<sup>GFP</sup> chimeras on day 4, their percentage as a percentage of total CD4<sup>+</sup> SPs rose steadily until day 8 (Fig 1.4A). T<sub>reg</sub> in these mice were present at a reduced percentage compared to the *Rag1*<sup>-/-</sup> host TetZap70

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FoxP3<sup>GFP</sup> chimeras (Fig 1.4A), with percentages comparable to that of the TetZap70 FoxP3<sup>GFP</sup> intact mice. Interestingly the frequency of cells expressing HuCD2 and therefore the transgene were on average 50% in both the TetZap70 FoxP3<sup>GFP</sup> host chimeras and intact mice. This is in both cases reduced compared to the *Rag1*<sup>-/-</sup> host TetZap70 FoxP3<sup>GFP</sup> chimeras which on average have 65% of DPs expressing the transgene (Fig 1.4B). TetZap70 FoxP3<sup>GFP</sup> host chimeras did however appear to have more consistent transgene induction compared to the intact mice. This suggests that the use of *Rag1*<sup>-/-</sup> chimeras is responsible for the more consistent transgene expression however it remains unclear what led to the increased number of cells expressing the transgene in these mice.

### **Lower T<sub>reg</sub> percentages in chimeras than constitutively dox fed mice**

Since *Rag1*<sup>-/-</sup> TetZap70 FoxP3<sup>GFP</sup> chimeras had more consistent transgene expression and slightly increased levels of thymic T<sub>reg</sub> we chose to continue further using *Rag1*<sup>-/-</sup> chimeras (TetZap70 FoxP3<sup>GFP</sup> chimeras here on in). To further characterise de novo T<sub>reg</sub> development TetZap70 FoxP3<sup>GFP</sup> chimeras in more detail, groups of mice were fed dox and analysed at days 0-8. Looking within the CD4<sup>+</sup> SP TCR<sup>Hi</sup> CD5<sup>Hi</sup> mature compartment, the first GFP<sup>+</sup> T<sub>reg</sub> appeared at day 4 with percentages steadily increasing until day 8 (Fig 1.5A&B). Interestingly when comparing thymic T<sub>reg</sub> percentages in the chimeras with WT controls, the size of the thymic FoxP3<sup>+</sup> T<sub>reg</sub> population plateau at a

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lower percentage of CD4<sup>+</sup> SPs than normally observed in a Bl/6 mouse (Fig 1.5C). This was lower than that observed in constitutively dox fed TetZap70 FoxP3<sup>GFP</sup> mice. We therefore characterised FoxP3<sup>+</sup> cells in TetZap70 FoxP3<sup>GFP</sup> mice by analysing expression of various T<sub>reg</sub> markers. TetZap70 FoxP3<sup>GFP</sup> mice or *Rag1*<sup>-/-</sup> chimeras were fed dox for 13 days and their thymic GFP<sup>+</sup> T<sub>reg</sub> analysed by FACs for the presence of T<sub>reg</sub> lineage markers. Expression of CTLA-4, GITR, GFP and CD5 on GFP<sup>+</sup> cells in TetZap70 FoxP3<sup>GFP</sup> chimeras were similar to those of GFP<sup>+</sup> T<sub>reg</sub> from FoxP3<sup>GFP</sup> mice (Fig 1.6). Interestingly T<sub>reg</sub> in the dox fed mice were almost all CD62L<sup>Hi</sup>, while the FoxP3<sup>GFP</sup> control contains both positive and negative populations (Fig 1.6). These results suggest that by day 13 T<sub>reg</sub> in the TetZap70FoxP3<sup>GFP</sup> mice display surface markers associated with the T<sub>reg</sub> lineage and can therefore appear to have undergone normal maturation. However we also saw a reduced percentage of the thymic T<sub>reg</sub> in TetZap70 FoxP3<sup>GFP</sup> chimeras when compared to both WT and constitutively dox fed TetZap70 mice. This suggests that the thymic T<sub>reg</sub> pool is not solely composed of de novo developed T<sub>reg</sub> and perhaps contains a recirculating T<sub>reg</sub> population.

### **Significant proportions of thymic FoxP3<sup>+</sup> T<sub>reg</sub> are recirculants.**

Next we looked in to the possibility that T<sub>reg</sub> recirculate back into the thymus from the periphery. T<sub>reg</sub> percentages in both TetZap70 FoxP3<sup>GFP</sup> chimeras and intact mice plateau lower than that normally observed in WT Bl/6 (Fig 1.5C). As

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TetZap70 FoxP3<sup>GFP</sup> mice have an empty periphery at these early time points, the lack of a recirculating population of T<sub>reg</sub> could explain the lower percentages observed in these mice. Congenically labelled lymph node cells were iv injected into un-induced TetZap70 FoxP3<sup>GFP</sup> mice and left for 3 weeks. The TetZap70 FoxP3<sup>GFP</sup> mice were then placed on dox for 6 days and their thymus analysed by FACs. The host CD45.1<sup>-</sup> compartment contained DN, DP and SP populations, indicative of normal thymic development. While donor CD45.1<sup>+</sup> cells comprised mature CD4<sup>+</sup> and CD8<sup>+</sup> SPs injected into the periphery 3 weeks previously (Fig 1.7A). Looking within the CD4<sup>+</sup> SP compartments approximately 0.5% of host and 4% of donor CD4<sup>+</sup> SPs cells are FoxP3<sup>+</sup> (Fig 1.7A). Gating on total FoxP3<sup>+</sup> cells; iv injected donor cells make up approximately 30% of thymic T<sub>reg</sub> while only comprising 6% of total CD4<sup>+</sup> SPs (Fig 1.7B). Host FoxP3<sup>+</sup> CD45.1<sup>+</sup> cells also contain increased frequencies of FoxP3<sup>+</sup> CD25<sup>Hi</sup> cells relative to FoxP3<sup>+</sup> CD45.1<sup>-</sup> donor cells (Fig 1.7B). Taken together this indicates that thymic T<sub>reg</sub> are not solely comprised of de novo developing nT<sub>reg</sub> but in fact significant proportions of T<sub>reg</sub> in the periphery are able to recirculate into the thymus. Further evidence of recirculation came from constitutively dox fed mice. TetZap70 FoxP3<sup>GFP</sup> mice were placed on dox from birth, then at 16 weeks of age removed from dox and placed onto normal food, completely turning off Zap70 expression. The thymus of these mice were then analysed by FACs 4 weeks later. Approximately 3.8% of mature thymic CD4<sup>+</sup> SPs in these mice were GFP<sup>+</sup> T<sub>reg</sub>. Interestingly nearly all of the GFP<sup>+</sup> T<sub>reg</sub> showed high levels of helios expression, a transcription factor reported to be a marker of thymic derived nT<sub>reg</sub> (Fig 1.7C) (A. M. Thornton et al. 2010). The presence of GFP<sup>+</sup> T<sub>reg</sub>

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in the absence of T-cell development, further indicates recirculation of T<sub>reg</sub> from the periphery into the thymus. It also casts some doubt over the use of helios as a marker of thymically derived nT<sub>reg</sub>.

### **GITR levels increase throughout T-cell development**

FoxP3<sup>+</sup> T<sub>reg</sub> are reported to develop from GITR<sup>Hi</sup> CD25<sup>Hi</sup> FoxP3<sup>-</sup> CD4<sup>+</sup> SP progenitor thymocytes. In order to identify this precursor population we analysed expression of these markers throughout T cell development in the TetZap70 FoxP3<sup>GFP</sup> chimeras. TetZap70 FoxP3<sup>GFP</sup> chimeras were fed dox for 3-6 days and the phenotype of the CD4<sup>+</sup> SPs analysed by FACs. Analysing GITR expression revealed that CD4<sup>+</sup> SP thymocytes initially were GITR<sup>Lo</sup>. GITR levels then begin to increase at day 4 continuing throughout the time course. GFP<sup>+</sup> T<sub>reg</sub> appear at day 4 and were GITR<sup>High</sup> (Fig 1.8A). Since CD25<sup>High</sup> GITR<sup>high</sup> FoxP3<sup>-</sup> cells are proposed to be enriched with T<sub>reg</sub> precursors we analysed CD25 and GFP expression levels specifically on GITR<sup>High</sup> CD4<sup>+</sup> SP thymocytes (Fig 1.8B). Gating specifically on GFP<sup>+</sup> CD4<sup>+</sup> SP GITR<sup>High</sup> cells (Fig 1.8 B) revealed that GFP<sup>+</sup> T<sub>reg</sub> develop both CD25<sup>High</sup> and CD25<sup>Low</sup>. The total GFP<sup>+</sup> compartment consisted of approximately half CD25<sup>High</sup> cells and half CD25<sup>Low</sup> cells. Analysing the total CD4<sup>+</sup> SP GFP<sup>-</sup> compartment; we found that CD25<sup>High</sup> GITR<sup>High</sup> population accumulate at d4, however CD25 expression is not confined exclusively to the GITR<sup>High</sup> compartment. There were also significant percentages of GITR<sup>High</sup> CD25<sup>Low</sup> cells present (Fig 1.8C). This data

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suggests the presence of a  $\text{GITR}^{\text{High}} \text{FoxP3}^- \text{T}_{\text{reg}}$  precursor population, however it remains unclear if the  $\text{T}_{\text{reg}}$  precursors are enriched in either the  $\text{CD25}^{\text{High}}$  or  $\text{CD25}^{\text{Low}} \text{GITR}^{\text{High}}$  compartment.

### **$\text{T}_{\text{reg}}$ develop from a $\text{GITR}^{\text{High}} \text{Nur77}^{\text{High}}$ precursor population**

Studies show that  $\text{T}_{\text{reg}}$  precursors are selected with a strong TCR signal. We therefore sort to identify this population within the  $\text{GITR}^{\text{High}}$  compartment. To do this, we took advantage of  $\text{Nr4a1}^{\text{GFP}}$  mice ( $\text{Nur77}^{\text{GFP}}$ ). Nur77 belongs to the nuclear receptor superfamily and is induced upon TCR signalling in  $\text{CD4}^+$  T cells, including immature thymocytes (Kashiwagi & Waldmann 2011). It has been shown in  $\text{Nur77}^{\text{GFP}}$  mice that strength of TCR signalling correlates with GFP expression (Moran, 2011). As  $\text{T}_{\text{reg}}$  precursors are proposed to be selected with a strong TCR signal,  $\text{Nur77}^{\text{GFP}}$  mice were analysed with the aim of identifying a  $\text{T}_{\text{reg}}$  precursor population within the  $\text{GITR}^{\text{High}}$  compartment.  $\text{TetZap70}$  mice crossed with  $\text{Nur77}^{\text{GFP}}$  mice, ( $\text{TetZap70 Nur77}^{\text{GFP}}$ ) were used to make bone marrow chimeras.  $\text{TetZap70 Nur77}^{\text{GFP}}$  chimeras were then fed dox and their thymocytes analysed by FACs. Cells were prefixed with 0.5% paraformaldehyde in order to maintain intracellular GFP, before being permeabilised and stained for FoxP3. At day 5, percentages of  $\text{FoxP3}^+$  cells in the thymus were comparable to those observed in  $\text{TetZap70 FoxP3}^{\text{GFP}}$  chimeras (Fig 1.9A). Gating specifically on  $\text{GFP}^+ \text{GITR}^{\text{Hi}}$  cells showed a 20-fold enrichment in  $\text{FoxP3}^+$  cells as well as a more modest enrichment in  $\text{CD25}^{\text{High}}$



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FoxP3<sup>-</sup> cells (Fig 1.9B). T<sub>reg</sub> are reported to develop following a strong defined TCR signal. We therefore analysed Nur77<sup>GFP</sup> expression in CD25<sup>Low</sup> GFP<sup>+</sup>, CD25<sup>Hi</sup> GFP<sup>+</sup> and CD25<sup>Hi</sup> GFP<sup>-</sup> cells. Interestingly Nur77<sup>GFP</sup> expression on CD25<sup>High</sup> FoxP3<sup>+</sup> cells and CD25<sup>Low</sup> FoxP3<sup>+</sup> cells was identical while the CD25<sup>High</sup> FoxP3<sup>-</sup> cells showed increased GFP expression levels (Fig 1.9C). This suggests the CD25<sup>Hi</sup> GFP<sup>-</sup> cells had received a stronger TCR signal than the GFP<sup>+</sup> CD25<sup>Hi</sup> T<sub>reg</sub>. Next in order to look at the relative maturation of the CD25<sup>Low</sup> GFP<sup>+</sup>, CD25<sup>Hi</sup> GFP<sup>+</sup> and CD25<sup>Hi</sup> GFP<sup>-</sup> populations we analysed HSA expression. HSA expression was highest in CD25<sup>High</sup> FoxP3<sup>-</sup> cells and reduced in CD25<sup>Low</sup> FoxP3<sup>+</sup> and CD25<sup>High</sup> FoxP3<sup>+</sup> cells respectively (Fig C1.9C). The above data calls into question whether the CD25<sup>High</sup> FoxP3<sup>-</sup> CD4<sup>+</sup> SP cells are the sole CD25<sup>Hi</sup> FoxP3<sup>+</sup> T<sub>reg</sub> precursor population, and suggests that CD25<sup>Low</sup> GFP<sup>+</sup> cells are also capable developing into CD25<sup>High</sup> FoxP3<sup>+</sup> T<sub>reg</sub>.

### **GFP<sup>+</sup> CD25<sup>Low</sup> cells can behave as precursors to CD25<sup>High</sup> GFP<sup>+</sup> cells**

One advantage of the TetZap70 system is the ability to view the first wave of T-cell development, and to not rely on observations made in adult mice in which T-cell populations are under homeostatic control. So having suggested that CD25<sup>Low</sup> FoxP3<sup>+</sup> CD4<sup>+</sup> SP thymocytes can function as precursors to CD25<sup>Hi</sup> FoxP3<sup>+</sup> CD4<sup>+</sup> SP cells, we hypothesised that these cells would develop prior to CD25<sup>Hi</sup> FoxP3<sup>+</sup> CD4<sup>+</sup> SP cells in our TetZap70 FoxP3<sup>GFP</sup> mice, followed by the appearance of a CD25<sup>Hi</sup> population. TetZap70 FoxP3<sup>GFP</sup> chimeras were placed

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on dox and their thymus analysed by FACs after 1-8 days. We determined the timing and quantified the abundance of both the CD25<sup>High</sup> and CD25<sup>Low</sup> T<sub>reg</sub>. Subsequently 3 days after dox feeding, when T<sub>reg</sub> first develop; they are almost exclusively CD25<sup>Low</sup>. As the time course progresses the GFP<sup>+</sup> CD25<sup>High</sup> population becomes more prominent, until percentages almost reach that of the CD25<sup>Low</sup> T<sub>reg</sub> (Fig 1.10A). The percentage of FoxP3<sup>-</sup> CD25<sup>High</sup> cells, reported to be enriched in T<sub>reg</sub> precursors (Chan-Wang Joaquim Lio & C.-S. Hsieh 2008), remain constant throughout the time course (Fig 1.10A). IL-2 is suggested to be the main signal driving the initial expression of Foxp3 in developing thymocytes. To investigate the role of IL-2 we sorted FoxP3<sup>-</sup> CD25<sup>High</sup>, FoxP3<sup>-</sup> CD25<sup>Low</sup> and FoxP3<sup>+</sup> CD25<sup>Low</sup> thymocytes. T<sub>reg</sub> were sorted from FoxP3<sup>GFP</sup> mice and cultured for 24 hours in vitro with or without IL-2. Sorted GFP<sup>+</sup> CD25<sup>Low</sup> cells up-regulated CD25 expression levels upon the addition of IL-2 (Fig 1.10B). Some GFP<sup>-</sup> CD25<sup>+</sup> cells also began to express GFP when cultured in media containing IL-2, however this was a modest increase and GFP expression levels remained lower than those observed in GFP<sup>+</sup> CD25<sup>Low</sup> cells. Addition of IL-2 had no effect on CD25<sup>Low</sup> GFP<sup>-</sup> cells (Fig 1.10B). Taken together these results suggest that substantial proportions of CD25<sup>Low</sup> FoxP3<sup>+</sup> cells can increase CD25 expression upon exposure to IL-2, becoming FoxP3<sup>+</sup> CD25<sup>High</sup> T<sub>reg</sub>. CD25<sup>High</sup> GFP<sup>-</sup> cells also appear able to contribute to the development of CD25<sup>High</sup> FoxP3<sup>+</sup> T<sub>reg</sub> however with CD25<sup>Low</sup> FoxP3<sup>+</sup> cells appearing prior to CD25<sup>High</sup> FoxP3<sup>+</sup> a precursor product relationship between the two cannot be excluded.

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### **GFP<sup>+</sup> CD25<sup>Low</sup> cells behave as highly efficient T<sub>reg</sub> precursors**

It remained possible that both CD25<sup>Low</sup> FoxP3<sup>+</sup> and CD25<sup>High</sup> FoxP3<sup>-</sup> thymocytes were precursors to FoxP3<sup>+</sup> CD25<sup>Hi</sup> T<sub>reg</sub>. In order to look at the relative efficiencies each of the CD25<sup>Low</sup> and CD25<sup>High</sup> precursor populations to differentiate into FoxP3<sup>+</sup> T<sub>reg</sub> we analysed GTR expression. As previously we sorted FoxP3<sup>-</sup> CD25<sup>High</sup>, FoxP3<sup>-</sup> CD25<sup>Low</sup> and FoxP3<sup>+</sup> CD25<sup>Low</sup> thymocytes from FoxP3<sup>GFP</sup> mice and cultured them with or without IL-2. We then analysed CD25 and GFP expression on GTR<sup>High</sup> cells after 24 hours. Sorted CD25<sup>Low</sup> GFP<sup>-</sup> cells contained very few, if any GTR<sup>High</sup> cells, in the presence or absence of IL-2. Upon IL-2 stimulation CD25<sup>High</sup> GFP<sup>-</sup> cells increased both their GTR and CD25 expression levels accompanied by modest levels of FoxP3 induction in the GTR<sup>High</sup> cells (Fig 1.11). The CD25<sup>Low</sup> GFP<sup>+</sup> cells increased GTR levels upon exposure to IL-2 with a corresponding increase in CD25 expression. These cells also maintained their GFP expression levels. Therefore in the presence of IL-2 both CD25<sup>High</sup> GFP<sup>-</sup> and CD25<sup>Low</sup> GFP<sup>+</sup> cells can differentiate into mature CD25<sup>High</sup> GFP<sup>+</sup> T<sub>reg</sub>.

### **T<sub>reg</sub> thymic egress is delayed compared to non-regulatory CD4<sup>+</sup> SPs**

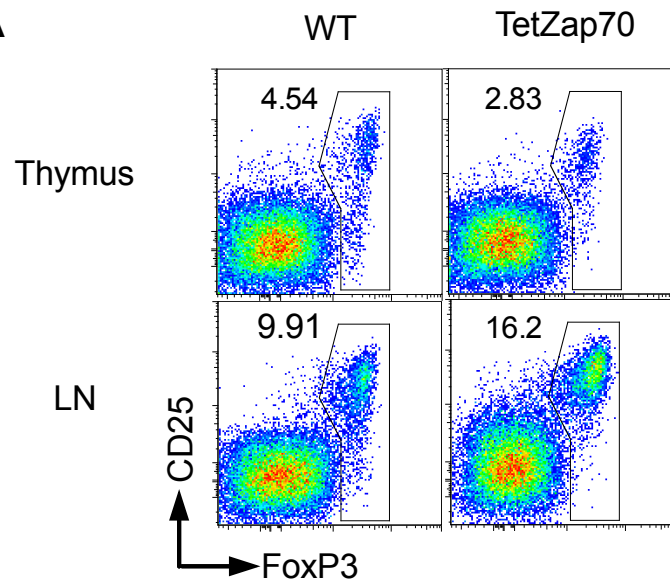
We next wanted to look at the timing of egress from the thymus by the FoxP3<sup>+</sup> compartment. Development of T<sub>reg</sub> in TetZap70 mice is delayed compared to

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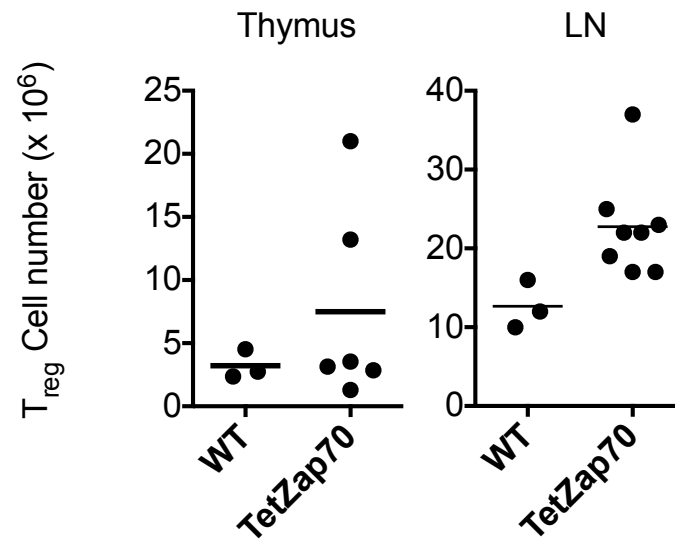
that of non-regulatory CD4<sup>+</sup> SP populations. We therefore wondered if there was also a delay in their egress from the thymus into the periphery. TetZap70 FoxP3<sup>GFP</sup> chimeras were fed dox their thymus and LNs analysed by FACs. Conventional CD4<sup>+</sup> SP T-cells appear in the LNs at day 5 with GFP<sup>+</sup> T<sub>reg</sub> appearing at day 10 (Fig 1.12A). There is therefore a significant delay in egress of the T<sub>reg</sub> compared to that of non-regulatory populations. Next we wondered if CD25 expression levels affected the cells ability to exit the thymus. We have shown substantial development of a GFP<sup>+</sup> CD25<sup>Low</sup> population in the thymus of TetZap70 FoxP3<sup>GFP</sup> mice (Fig1.5A). However T<sub>reg</sub> are usually associated with having a predominantly CD25<sup>High</sup> FoxP3<sup>+</sup> phenotype. At day 10 in our TetZap70 FoxP3<sup>GFP</sup> chimeras we see substantial percentages of both CD25<sup>High</sup> and CD25<sup>Low</sup> T<sub>reg</sub> in both the thymus and periphery (Fig 1.12B). Our data therefore suggests that the CD25<sup>Low</sup> GFP<sup>+</sup> and CD25<sup>High</sup> GFP<sup>+</sup> T<sub>reg</sub> are equally capable of emigrating from the thymus to the periphery and that this egress is approximately 5 days after that of the CD4<sup>+</sup>SP non-regulatory populations.

## 1.1

**A**



**B**

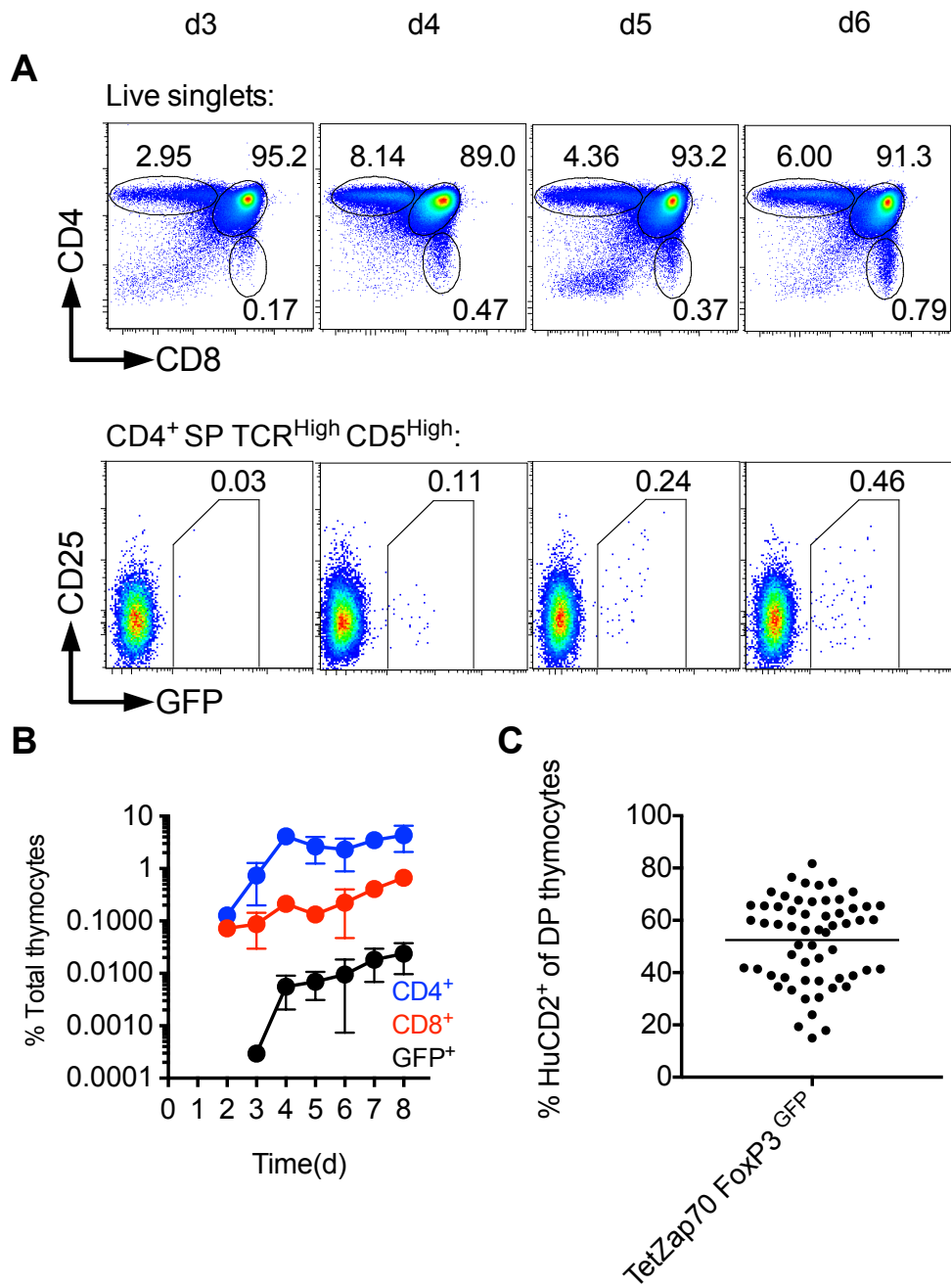


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### Figure 1.1 Characterisation of doxycycline fed TetZap70 mice

TetZap70 FoxP3<sup>GFP</sup> mice were fed doxycycline from birth followed by analysis of thymus and LNs by FACs at approximately 16 weeks of age. **A:** Representative FACs plots of CD4<sup>+</sup> SP TCR<sup>Hi</sup> CD5<sup>Hi</sup> cells from thymus (upper panels) and LNs (Lower panels) in WT Bl/6 (left panels) and TetZap70 FoxP3<sup>GFP</sup> mice (right panels). **B:** Absolute T<sub>reg</sub> cell numbers (CD4<sup>+</sup> TCR<sup>Hi</sup> CD5<sup>Hi</sup> FoxP3<sup>+</sup>) in Thymus (left panel) and LNs (right panel) of WT Bl/6 (n=3) and TetZap70 (n=6) mice.

## 1.2



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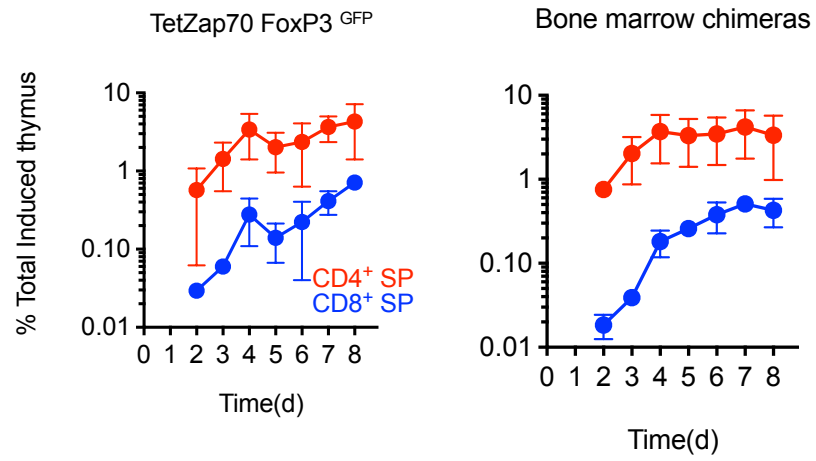
### Figure 1.2 Characterisation of doxycycline fed TetZap70 FoxP3<sup>GFP</sup> mice

TetZap70 FoxP3<sup>GFP</sup> mice were fed doxycycline for 3-6 days followed by analysis of the thymus by FACs. **A:** FACs plots showing CD4 Vs CD8 expression (upper panels) and CD25 Vs GFP expression (lower panels) in CD4<sup>+</sup> SP TCR<sup>Hi</sup> CD5<sup>Hi</sup> thymocytes of TetZap70 FoxP3<sup>GFP</sup> mice fed dox for 3-6 days. **B:** Time course of GFP<sup>+</sup> cells (black circles) CD8<sup>+</sup> SPs (red circles) and CD4<sup>+</sup> SPs (blue circles) as a percentage of total thymocytes after various durations of feeding dox. **C:** %HUCD2 reporter expression assessed by FACs when gated on CD4<sup>+</sup> CD8<sup>+</sup> DP thymocytes in TetZap70 FoxP3<sup>GFP</sup> mice fed dox for 3-6 days (n=59).

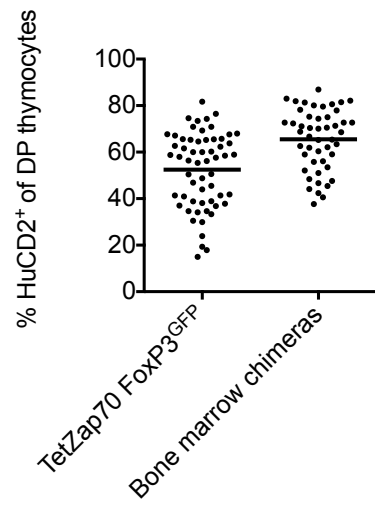


### 1.3

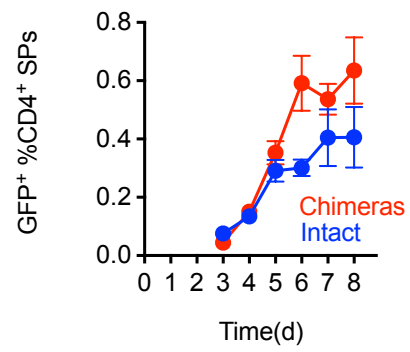
#### A



#### B



#### C



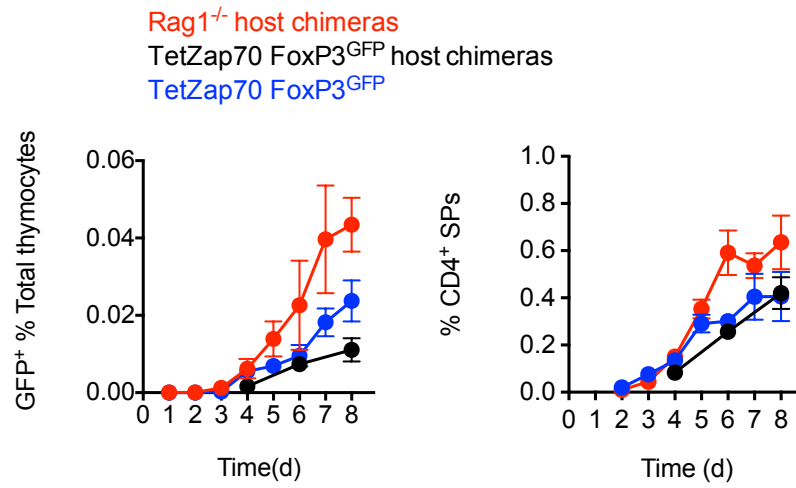
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**Figure 1.3 TetZap70 FoxP3<sup>GFP</sup> bone marrow chimeras.**

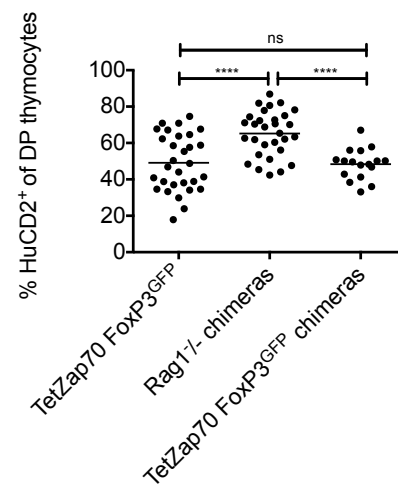
Rag1<sup>-/-</sup> mice were irradiated and injected with 5M TetZap70 FoxP3<sup>GFP</sup> bone marrow cells iv. Bone marrow was harvested as described in materials and methods. Chimeras were left for 6 weeks to reconstitute before being placed on dox along with TetZap70 FoxP3<sup>GFP</sup> mice. **A:** Time course for TetZap70 FoxP3<sup>GFP</sup> mice (left panel) and TetZap70 FoxP3<sup>GFP</sup> chimeras (right panel) showing CD8<sup>+</sup> SPs (red circles) and CD4<sup>+</sup> SPs (blue circles) as a percentage of total thymocytes after various durations of feeding dox. **C:** %HUCD2 reporter expression assessed by FACs when gated on CD4<sup>+</sup> CD8<sup>+</sup> DP thymocytes in TetZap70 FoxP3<sup>GFP</sup> mice (n=59) and TetZap70 FoxP3<sup>GFP</sup> chimeras (n=48) fed dox for 2-8 days.

## 1.4

**A**



**B**

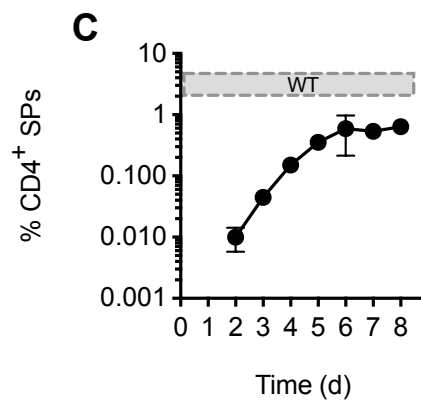
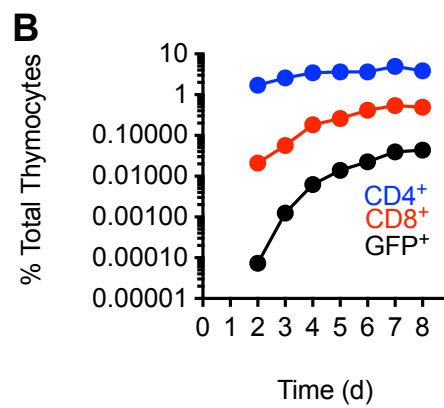
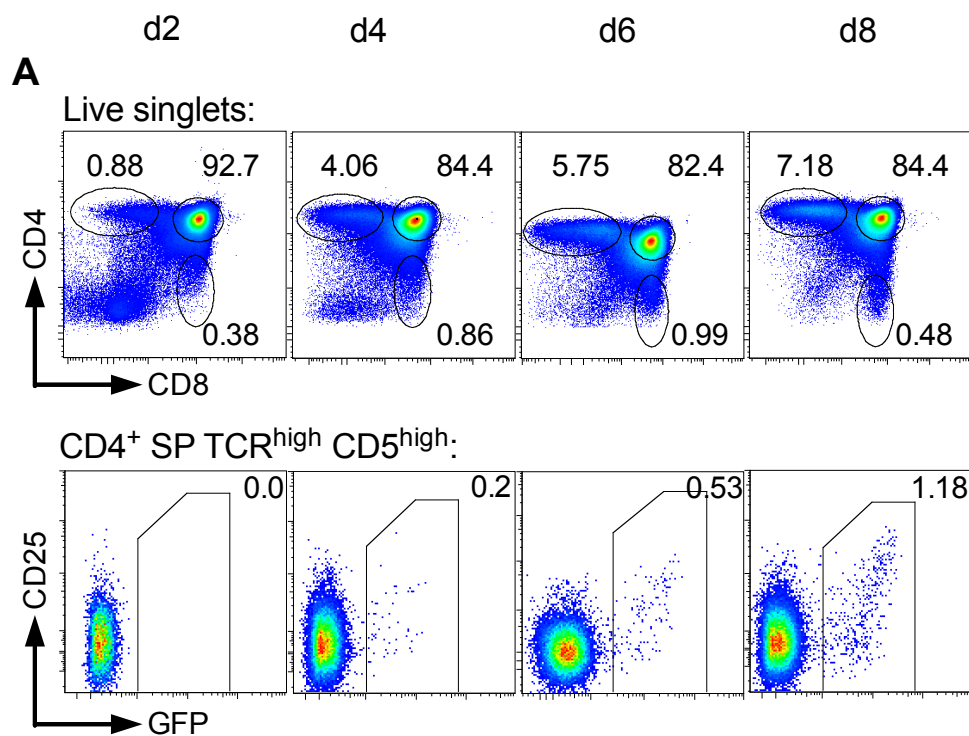


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**Figure 1.4 TetZap70 FoxP3<sup>GFP</sup> bone marrow chimeras.**

Rag1<sup>-/-</sup> or TetZap70 FoxP3<sup>GFP</sup> mice were irradiated and injected with 5M TetZap70 FoxP3<sup>GFP</sup> bone marrow cells iv. Bone marrow was harvested as described in materials and methods. Chimeras were left for 6 weeks to reconstitute before being placed on dox along with TetZap70 FoxP3<sup>GFP</sup> mice. **A:** Time course for TetZap70 FoxP3<sup>GFP</sup> mice (blue circles) and TetZap70 FoxP3<sup>GFP</sup> host chimeras (black circles) and Rag1<sup>-/-</sup> host chimeras (red circles) showing TCR<sup>Hi</sup> CD5<sup>Hi</sup> CD4<sup>+</sup> SP GFP<sup>+</sup> cells as a percentage of total thymocytes (left panel) and as a percentage of TCR<sup>Hi</sup> CD5<sup>Hi</sup> CD4<sup>+</sup> SPs (right panel), fed dox from 1-8 days. **B:** %HUCD2 reporter expression assessed by FACs when gated on CD4<sup>+</sup> CD8<sup>+</sup> DP thymocytes in TetZap70 FoxP3<sup>GFP</sup> mice (n=59), TetZap70 FoxP3<sup>GFP</sup> host chimeras (n=17) and Rag1<sup>-/-</sup> host chimeras (n=48) fed dox for 2-8 days.

1.5



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**Figure 1.5 T<sub>reg</sub> development in TetZap70 FoxP3<sup>GFP</sup> bone marrow chimeras.**

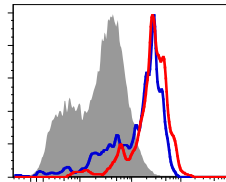
Rag1<sup>-/-</sup> mice were irradiated and injected with 5M TetZap70 FoxP3<sup>GFP</sup> bone marrow cells iv. Bone marrow was harvested as described in materials and methods. Chimeras were left for 6 weeks to reconstitute before being placed on dox for 2-8 days. **A:** FACs plots from the thymus of TetZap70 FoxP3<sup>GFP</sup> bone marrow chimeras fed dox for 2-8 days. Plots show live singlets (upper panels) and CD4<sup>+</sup> SP TCR<sup>Hi</sup> CD5<sup>Hi</sup> cells (lower panels) **B:** Time course for TetZap70 FoxP3<sup>GFP</sup> chimeras fed dox for 2-8 days showing TCR<sup>Hi</sup> CD5<sup>Hi</sup> CD4<sup>+</sup> SPs (blue circles) TCR<sup>Hi</sup> CD5<sup>Hi</sup> CD8<sup>+</sup> SPs (red circles) and TCR<sup>Hi</sup> CD5<sup>Hi</sup> CD4<sup>+</sup> SP GFP<sup>+</sup> (black circles) as a percentage of total thymocytes. **C:** Time course for TetZap70 FoxP3<sup>GFP</sup> chimeras fed dox for 2-8 days showing TCR<sup>Hi</sup> CD5<sup>Hi</sup> CD4<sup>+</sup> SP GFP<sup>+</sup> (black circles) as a percentage of TCR<sup>Hi</sup> CD5<sup>Hi</sup> CD4<sup>+</sup> SPs, compared to percentages normally observed in FoxP3<sup>GFP</sup> mice (range of n=4).

## 1.6

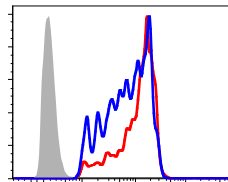
d13 TetZap70 FoxP3<sup>GFP</sup>

d13 FoxP3<sup>GFP</sup>

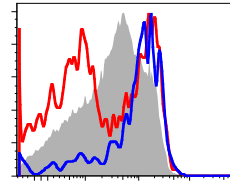
Naive CD4<sup>+</sup> SP



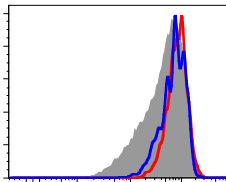
→ CTLA-4



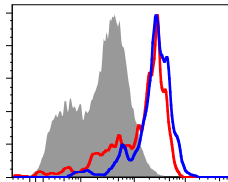
→ GFP



→ CD62L



→ CD5



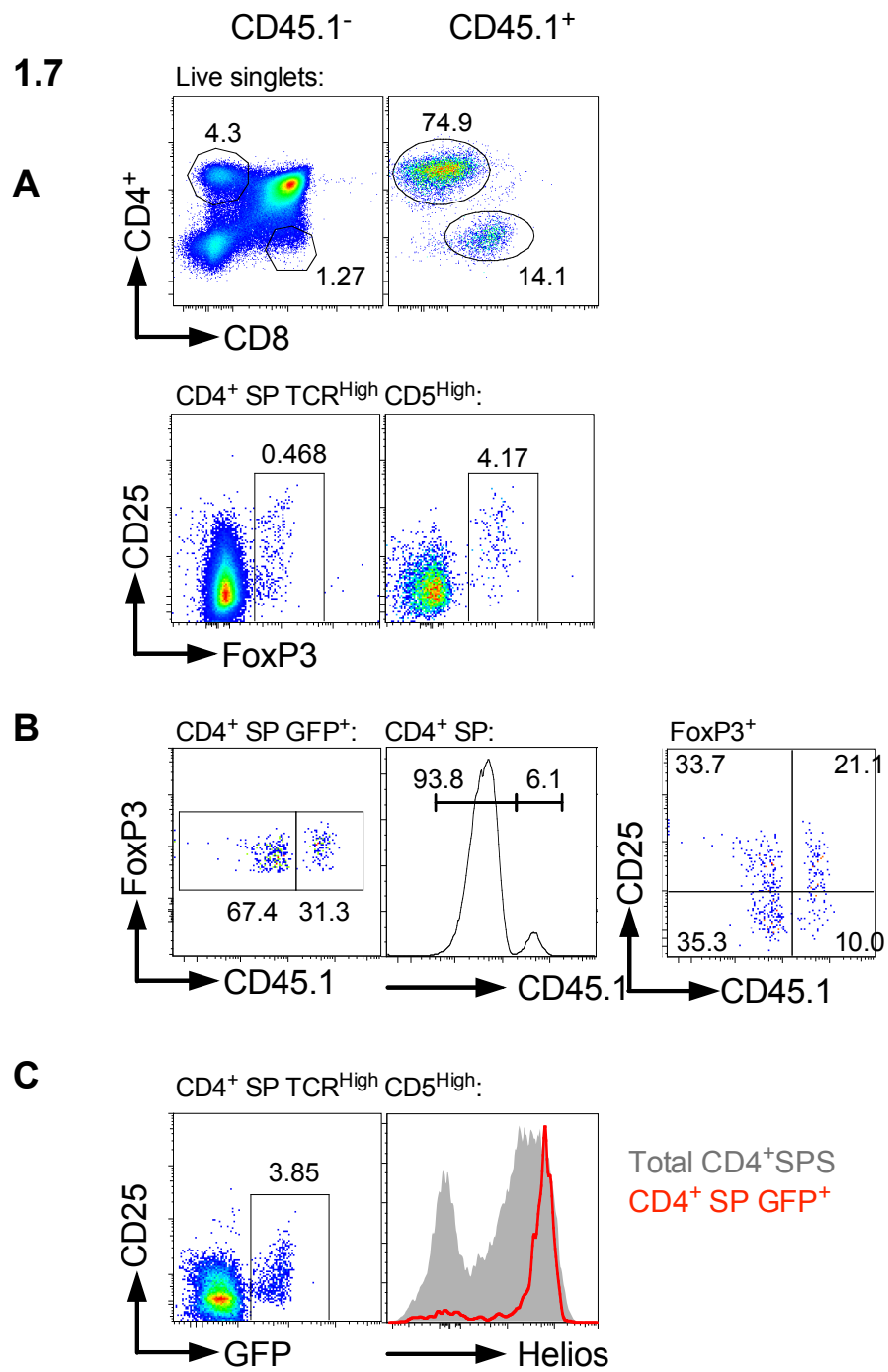
→ GITR

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**Figure 1.6 Characterisation of T<sub>reg</sub> in TetZap70 FoxP3<sup>GFP</sup> mice.**

TetZap70 FoxP3<sup>GFP</sup> mice were fed dox for 13 days prior to their thymus being analysed by FACs for expression of CTLA-4, CD62L, CD5 and GITR on TCR<sup>Hi</sup> CD5<sup>Hi</sup> CD4<sup>+</sup> SP GFP<sup>+</sup> T<sub>reg</sub> (blue) and compared to that of TCR<sup>Hi</sup> CD5<sup>Hi</sup> CD4<sup>+</sup> SP GFP<sup>+</sup> T<sub>reg</sub> from FoxP3<sup>GFP</sup> mice (red) and TCR<sup>Hi</sup> CD5<sup>Hi</sup> CD4<sup>+</sup> SP CD44<sup>Lo</sup> CD25<sup>Lo</sup> naïve cells from FoxP3<sup>GFP</sup> mice (grey)





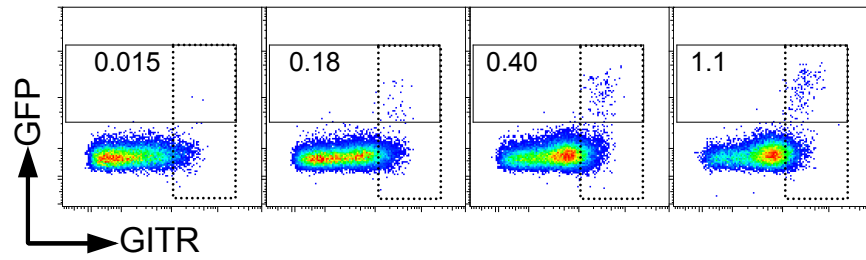
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**Figure 1.7 After thymic egress  $T_{reg}$  recirculate back to the thymus.**

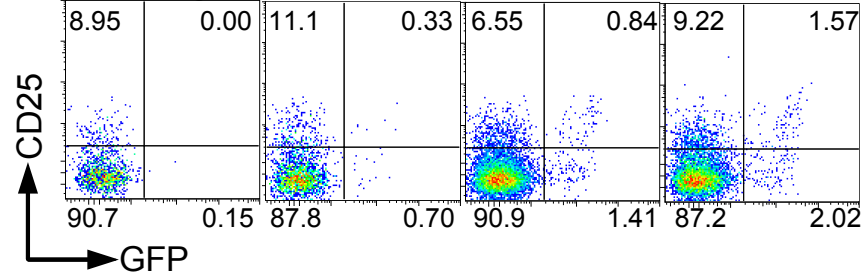
Rag1<sup>-/-</sup> mice were irradiated and injected with 5M TetZap70 FoxP3<sup>GFP</sup> bone marrow cells iv. Bone marrow was harvested as described in materials and methods. Chimeras were left for 6 weeks to reconstitute before being injected with 10M CD45.1 LN cells. After 4 weeks mice were fed dox for 6 days and their thymus analysed by FACs **A:** FACs plots showing CD4 and CD8 expression on live singlets (top panel), TetZap70 FoxP3<sup>GFP</sup> cells are CD45.1<sup>-</sup> (left column) while injected Bl/6 LN cells are CD45.1<sup>+</sup> (right column). TCR<sup>Hi</sup> CD5<sup>Hi</sup> CD4<sup>+</sup> SP cells were then analysed for FoxP3 and CD25 expression (bottom panel). **B:** Gating specifically on the TCR<sup>Hi</sup> CD5<sup>Hi</sup> CD4<sup>+</sup> SP GFP<sup>+</sup> compartment, cells were analysed for CD45.1, FoxP3 and CD25 expression (left and right) while TCR<sup>Hi</sup> CD5<sup>Hi</sup> CD4<sup>+</sup> SPs were analysed for CD45.1 expression also (middle). TetZap70 FoxP3<sup>GFP</sup> mice were fed dox from birth, then at approximately 16 weeks of age fed on normal dry diet for 4 weeks. **C:** FACs plot showing CD25 and GFP expression on TCR<sup>Hi</sup> CD5<sup>Hi</sup> CD4<sup>+</sup> SP cells (left). TCR<sup>Hi</sup> CD5<sup>Hi</sup> CD4<sup>+</sup> SPs (grey) and TCR<sup>Hi</sup> CD5<sup>Hi</sup> CD4<sup>+</sup> SP GFP<sup>+</sup> cells (red) were further analysed for helios expression (histogram right).

1.8      d3                      d4                      d5                      d6

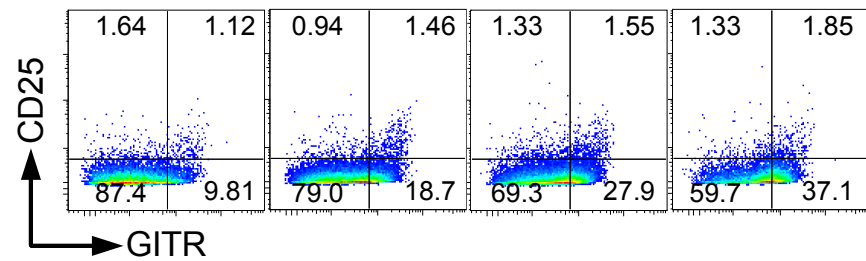
**A**    CD4<sup>+</sup> SP gated:



**B**    GITR<sup>High</sup> gated:



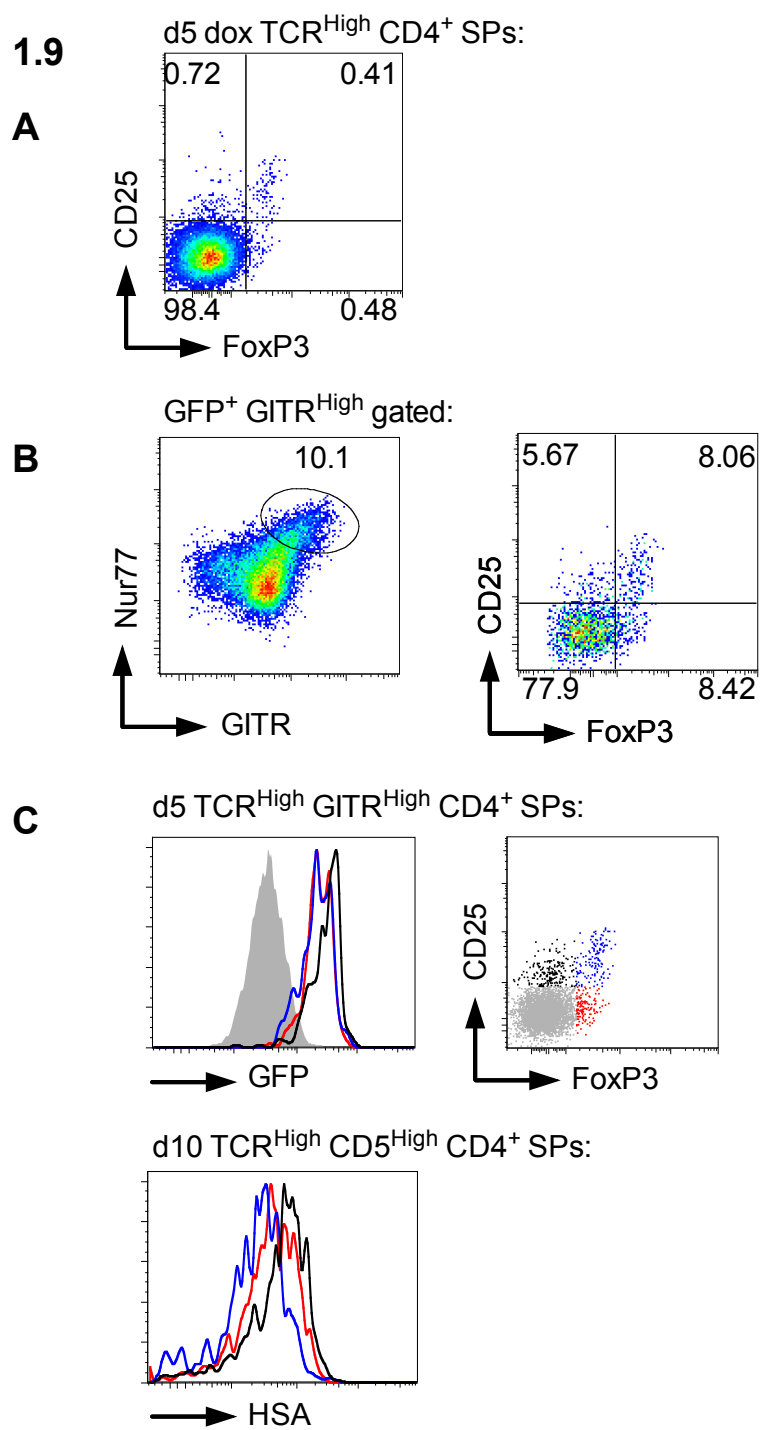
**C**    GFP<sup>-</sup> gated:



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### Figure 1.8 Identification of a GITR<sup>Hi</sup> FoxP3<sup>-</sup> T<sub>reg</sub> precursor

Rag1<sup>-/-</sup> mice were irradiated and injected with 5M TetZap70 FoxP3<sup>GFP</sup> bone marrow cells iv. Bone marrow was harvested as described in materials and methods. Chimeras were left for 6 weeks to reconstitute before being placed on dox for 3-6 days. **A:** Representative FACS plots from the thymus of TetZap70 FoxP3<sup>GFP</sup> bone marrow gated on total TCR<sup>Hi</sup> CD5<sup>Hi</sup> CD4<sup>+</sup> SPs **B:** Representative FACS plots from the thymus of TetZap70 FoxP3<sup>GFP</sup> bone marrow gated on total GITR<sup>Hi</sup> TCR<sup>Hi</sup> CD5<sup>Hi</sup> CD4<sup>+</sup> SPs (gating illustrated in dashed box in A) **C:** Representative FACS plots from the thymus of TetZap70 FoxP3<sup>GFP</sup> bone marrow gated on total TCR<sup>Hi</sup> CD5<sup>Hi</sup> CD4<sup>+</sup> SPs GFP<sup>-</sup> cells.



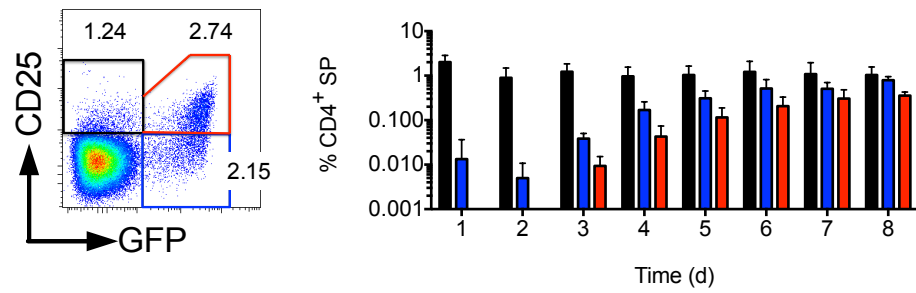
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### Figure 1.9 Identification of a GITR<sup>Hi</sup> FoxP3<sup>-</sup> T<sub>reg</sub> precursor

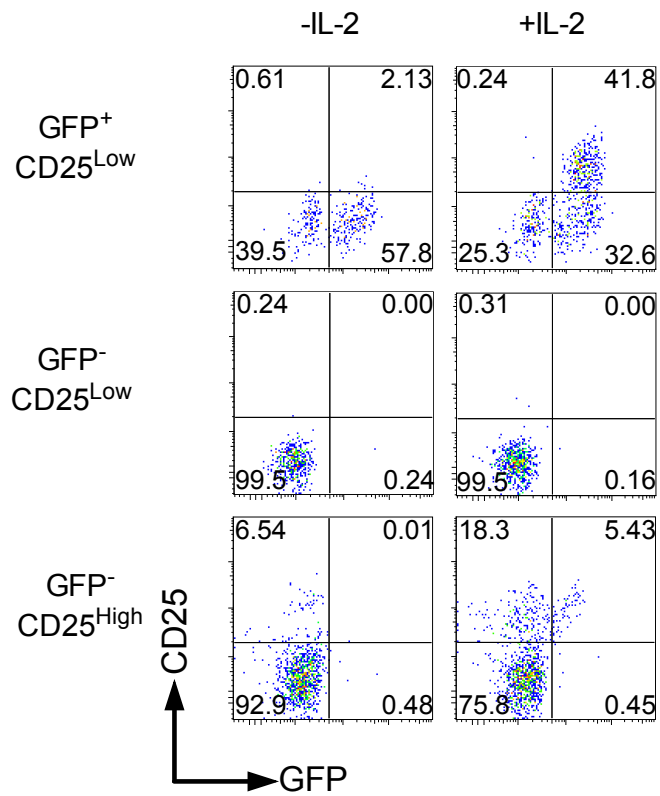
TetZap70 FoxP3<sup>GFP</sup> mice were crossed with Nur77<sup>GFP</sup> mice. The resulting TetZap70 Nur77<sup>GFP</sup> mice were fed dox and their thymus analysed by FACs after 5&10 days **A:** Representative FAC plot from the thymus of a d5 TetZap70 Nur77<sup>GFP</sup> mouse. Showing FoxP3 and CD25 expression within the CD4<sup>+</sup> SP TCR<sup>Hi</sup> compartment **B:** Representative FACs plots from d5 TetZap70 Nur77<sup>GFP</sup> mouse, CD4<sup>+</sup> SP TCR<sup>Hi</sup> (left panel), further gated on GFP<sup>Hi</sup> GITR<sup>Hi</sup> cells showing FoxP3 and CD25 expression (right panel). **C:** Representative FACs plots from d5 dox fed TetZap70 Nur77<sup>GFP</sup> mice gated on TCR<sup>Hi</sup> GITR<sup>Hi</sup> CD4<sup>+</sup>SPs. FoxP3<sup>+</sup> CD25<sup>Lo</sup> cells (red) FoxP3<sup>+</sup> CD25<sup>Hi</sup> cells (blue) FoxP3<sup>-</sup> CD25<sup>Hi</sup> cells (black) and FoxP3<sup>-</sup> CD25<sup>Lo</sup> (grey) (top right) compared for levels of GFP expression (top left) and d10 dox fed TetZap70 Nur77<sup>GFP</sup> same populations as (top right) but compared for HSA expression (bottom panel).

## 1.10

**A**



**B**



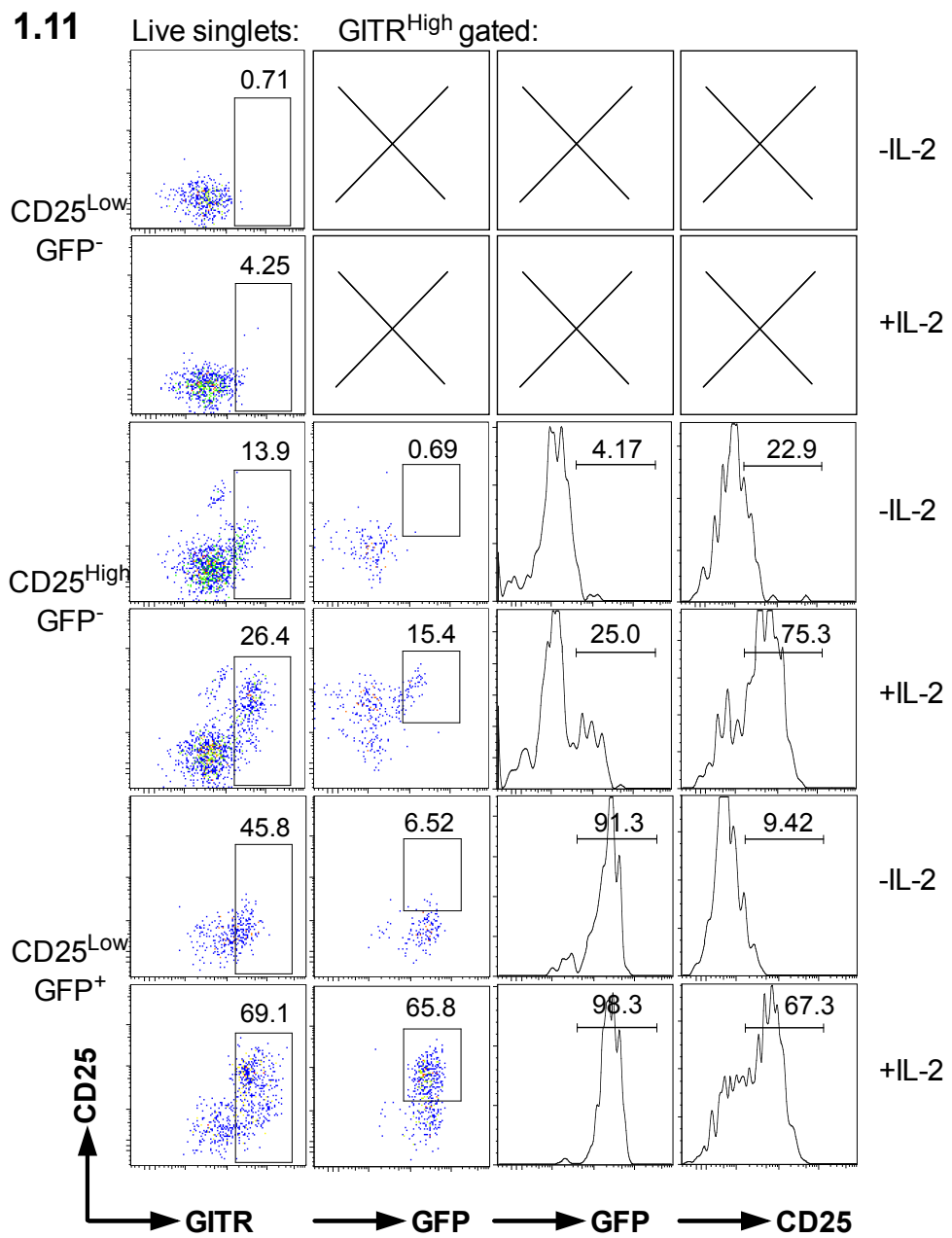
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**Figure 1.10 IL-2 culture of CD25<sup>Hi</sup> and CD25<sup>Lo</sup> T<sub>reg</sub> precursor populations**

Rag1<sup>-/-</sup> mice were irradiated and injected with 5M TetZap70 FoxP3<sup>GFP</sup> bone marrow cells iv. Bone marrow was harvested as described in materials and methods. Chimeras were left for 6 weeks to reconstitute before being placed on dox for 1-8 days. **A:** (left panel) Representative FACS plot of gating strategy employed when studying development of TCR<sup>Hi</sup> CD5<sup>Hi</sup> CD4<sup>+</sup> SP<sup>-</sup> CD25<sup>Hi</sup> GFP<sup>-</sup> (black), CD25<sup>Lo</sup> GFP<sup>+</sup> (blue) or CD25<sup>Hi</sup> GFP<sup>+</sup> cells (red) in TetZap70 FoxP3<sup>GFP</sup> chimeras, illustrated using FoxP3<sup>GFP</sup> thymus. (right panel) Histogram showing the abundance of the populations detailed above after various durations of dox feeding, as a percentage of TCR<sup>Hi</sup> CD5<sup>Hi</sup> CD4<sup>+</sup> SPs.

CD25<sup>Lo</sup> FoxP3<sup>+</sup> T<sub>reg</sub> are able to increase CD25 expression after culture with IL-2. TCR<sup>Hi</sup> CD5<sup>Hi</sup> CD4<sup>+</sup> SP<sup>-</sup> CD25<sup>Hi</sup> GFP<sup>-</sup>, CD25<sup>Lo</sup> GFP<sup>+</sup> or CD25<sup>Lo</sup> GFP<sup>-</sup> cells were sorted from FoxP3<sup>GFP</sup> mice (n=4) and cultured with or without 10ng/μl IL-2 in RPMI for 48 hours. **B:** Representative FACS plots of cultured cells described above which were analysed by FACS for CD25 and GFP expression after 48 hours.





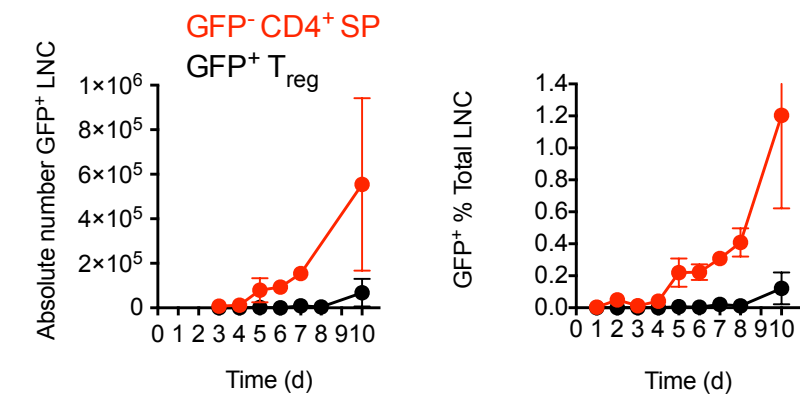
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**Figure 1.11 IL-2 culture of CD25<sup>Hi</sup> and CD25<sup>Lo</sup> T<sub>reg</sub> precursor populations**

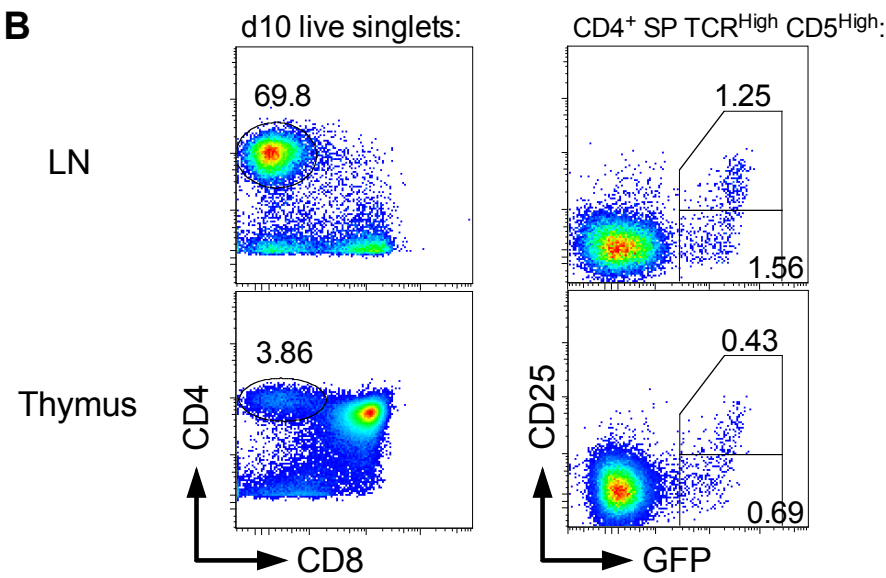
TCR<sup>Hi</sup> CD5<sup>Hi</sup> CD4<sup>+</sup> SP<sup>-</sup> CD25<sup>Hi</sup> GFP<sup>-</sup>, CD25<sup>Lo</sup> GFP<sup>+</sup> or CD25<sup>Lo</sup> GFP<sup>-</sup> cells were sorted from FoxP3<sup>GFP</sup> mice (n=4) and cultured with or without 10ng/μl IL-2 in RPMI. Cells were then analysed by FACs after 48 hours. The various sorted and cultured populations were analysed for CD25 and GITR expression by FACs (first left). Cultured cells were further gated for high GITR expression and analysed for GFP and CD25 expression (Second left). GITR<sup>Hi</sup> cells were then analysed for GFP (second right) and CD25 expression (first right).

1.12

A



B



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**Figure 1.12 T<sub>reg</sub> Thymic egress is delayed relative to conventional T-cells**

Rag1<sup>-/-</sup> mice were irradiated and injected with 5M TetZap70 FoxP3<sup>GFP</sup> bone marrow cells iv. Bone marrow was harvested as described in materials and methods. Chimeras were left for 6 weeks to reconstitute before being placed on dox for 0-10 days and having their LNs analysed by FACs. **A:** Graph of TCR<sup>Hi</sup> CD5<sup>Hi</sup> CD4<sup>+</sup> SP GFP<sup>+</sup> absolute cell numbers in LNs (left panel), and as a percentage of total LN cells (right panel). Data taken from the LNs of TetZap70 FoxP3<sup>GFP</sup> mice fed dox from 0-10 days. **B:** Representative FACs plots from d10 TetZap70 FoxP3<sup>GFP</sup> mice showing live singlets (left panel) analysed for CD4 and CD8 expression, and TCR<sup>Hi</sup> CD5<sup>Hi</sup> CD4<sup>+</sup> SPs (right panel) analysed for CD25 and GFP expression. Cells taken from the LNs (top panels) and Thymus (bottom panels).

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## Discussion

During thymocyte development, thymic progenitors interpret TCR dependant signals and commit to a variety of T-cell lineages.  $T_{reg}$  are thought to be selected by self-antigens on a TCR signal strength between that needed for positive and negative selection; they are therefore thought to be slightly auto-reactive. In this study we found that FoxP3 expression first occurs in thymocytes 4 days after positive selection, approximately 3 days after the first appearance of conventional  $CD4^+$  SPs, which develop on day 1 (Fig 1.2). This study therefore identifies a relative delay in FoxP3 expression compared to other lineage defining transcription factors, however both the signals involved and the reason for this delay still remain unclear.

Previous studies have focussed on the neonatal thymus in order to observe the timing of the first wave of  $T_{reg}$  development. There are reports of thymic  $T_{reg}$  being present in neonates 3 days after birth, broadly agreeing with our findings (Cheng et al. 2013). However it can be difficult to draw comparisons between the timing in neonates where Zap70 is under endogenous control and in our *TetZap70 FoxP3<sup>GFP</sup>* mice where development is arrested at the  $CD4^+ CD8^+$  DP stage of development. We can however confirm a delay in the development of

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$T_{reg}$  compared to that of non-regulatory populations in adult mice whereas previously this had only been confirmed in neonates. In adult FoxP3<sup>GFP</sup> mice, many reports have shown GFP expression confined primarily to the medulla (Liston et al. 2008). Pharmacologic blockade of migration into the medulla from the cortex leads to an accumulation of GFP<sup>+</sup> cells in the cortex. This perhaps suggests  $T_{reg}$  receive a signal to differentiate in the cortex followed by a subsequent delay, before expressing FoxP3 in the medulla. Our data is consistent with this idea, as it has been shown that un-induced TetZap70 thymocytes are arrested in the cortex and are all CD4<sup>+</sup> CD8<sup>+</sup> DPs (White et al. 2010). Only when mice are placed on dox are CD4<sup>+</sup> SPs found located in the medulla. It therefore remains possible  $T_{reg}$  receive a cortical signal to differentiate but FoxP3 expression is delayed, only becoming detectable in the medulla 2-3 days after CD4<sup>+</sup> SPs develop.

Since  $T_{regs}$  were first discovered in the late 90s their thymic development has been the focus of much research. It has been proposed that the study of their thymic differentiation would be hugely simplified by the discovery of an immediate precursor that has not yet differentiated. Our data suggests that  $T_{reg}$  develop from a GITR<sup>High</sup> FoxP3<sup>-</sup> precursor population which has undergone strong TCR signalling (Fig 1.8 & 1.9). However it is currently unclear if this population is enriched in the CD25<sup>High</sup> FoxP3<sup>-</sup> compartment as stated by the current literature, or in fact develop from thymocytes with a broad range of CD25 expression. Currently the favoured model for  $T_{reg}$  development is the 2 step model (Chan-Wang Joaquim Lio & C.-S. Hsieh 2008). This model describe

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$T_{reg}$  progenitors undergoing strong TCR signalling leading to CD25 up regulation, this  $CD25^{High}$   $FoxP3^{-}$  precursor population then respond to IL-2 which drives FoxP3 expression. This model is based on the observation that when sorted and transferred to WT hosts,  $CD25^{High}$   $CD4^{+}$  SPs more efficiently express FoxP3 than  $CD25^{Low}$   $CD4^{+}$  SPs. In support of this model, we show that when sorting  $CD25^{High}$  and  $CD25^{Low}$   $GFP^{-}$  cells and culturing them with IL-2, the  $CD25^{High}$  cells give rise to  $CD25^{High}$   $GFP^{+}$   $T_{reg}$  much more efficiently than their  $CD25^{Low}$  counterparts. When culturing  $CD25^{Low}$   $GFP^{-}$  cells with IL-2 we see no rise in GFP or GITR expression whatsoever (Fig 1.11). These observations would seem to further support a 2 step model for  $T_{reg}$  development where  $CD25^{High}$   $FoxP3^{-}$  cells act as  $T_{reg}$  precursors. Although we see  $CD25^{High}$   $GFP^{-}$  cells acting as precursors upon culture with IL-2, we found  $CD25^{Low}$   $GFP^{+}$  do so much more efficiently (Fig 1.11). Upon exposure to IL-2  $CD25^{Low}$   $GFP^{+}$  cells increase both GITR and CD25 expression, at similar levels to that undergone by the  $CD25^{High}$   $GFP^{-}$  cells (Fig 1.11). However this CD25 expression is accompanied by a relatively modest level of GFP expression in the  $CD25^{High}$   $GFP^{-}$  cells. The cultured  $CD25^{Low}$  cells however were already expressing GFP when sorted from  $FoxP3^{GFP}$  mice so this could be somewhat of an unfair comparison. Also previous studies have suggested that not all  $CD25^{High}$  cells function as precursors, only cells transitioning from an IL-7 to an IL-2 dependency (Chan-Wang Joaquim Lio & C.-S. Hsieh 2008). It would therefore perhaps be expected that not all of these cells differentiate when stimulated with IL-2 in our culture experiments. It therefore remains possible that  $T_{reg}$  develop via either a  $CD25^{High}$  or  $CD25^{Low}$  precursor population, however our data

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suggests that the CD25<sup>High</sup> FoxP3<sup>-</sup> cells may make a more minor contribution to T<sub>reg</sub> development than previously thought.

During the TetZap70 FoxP3<sup>GFP</sup> time courses we see CD25<sup>Low</sup> FoxP3<sup>+</sup> cells developing prior to CD25<sup>High</sup> FoxP3<sup>+</sup> cells (Fig 1.10). However it remains unclear if these represent a precursor population to the CD25<sup>High</sup> GFP<sup>+</sup> T<sub>reg</sub> in vivo or alternate T<sub>reg</sub> lineage all together. We considered the possibility of an alternative model for T<sub>reg</sub> development, where T<sub>reg</sub> solely develop via a CD25<sup>Low</sup> FoxP3<sup>+</sup> precursor population and then up regulate their CD25 expression levels as they develop further. In support of this model we see identical Nur77 expression levels on CD25<sup>Low</sup> and CD25<sup>High</sup> FoxP3<sup>+</sup> cells, suggesting they have received a similar strength of TCR signal, while CD25<sup>High</sup> FoxP3<sup>-</sup> cells show increased Nur77 expression compared to the FoxP3 positive populations. As Nur77 is an established marker of TCR signalling strength and thought to be relatively stable, it is difficult to comprehend a reason why expression levels would drop as precursors differentiate into T<sub>reg</sub>. CD25<sup>High</sup> FoxP3<sup>-</sup> cells also express high levels of HSA suggesting they are immature relative to both CD25<sup>Low</sup> FoxP3<sup>+</sup> cells and CD25<sup>High</sup> FoxP3<sup>+</sup> T<sub>reg</sub> which exhibit very low HSA levels (Fig 1.9C). Taken together these observations suggest CD25<sup>High</sup> FoxP3<sup>-</sup> thymocytes have undergone strong TCR signalling at the very early HSA high stage of development perhaps questioning their role as precursors to the CD25<sup>High</sup> T<sub>reg</sub>, perhaps instead representing T-cells on the cusp of negative selection. Increased Nur77 expression on these CD25<sup>High</sup> FoxP3<sup>-</sup> cells has been observed by previous studies but only compared to the T<sub>reg</sub> compartment as a



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whole, and not the CD25<sup>Low</sup> T<sub>reg</sub> (Moran, 2011). In fact very few studies to date investigate a role for the CD25<sup>Low</sup> compartment. But Identical Nur77 expression coupled with lower HSA expression and their ability to increase CD25 levels upon exposure to IL-2 in vitro suggests there is a strong possibility of a precursor product relationship between these and the CD25<sup>High</sup> FoxP3<sup>+</sup> T<sub>reg</sub>. This linear model for T<sub>reg</sub> development would be contrary to both the 2 step model and studies in neonatal mice. In neonates T<sub>reg</sub> are reported to develop predominantly CD25<sup>High</sup> 3 days after birth (Cheng et al. 2013)(Fontenot et al. 2005). The discrepancy between CD25 expression levels in our TetZap70 system and neonatal mice could be for a number of reasons. Firstly in neonatal mice Zap70 expression is under endogenous control, however Zap70 in TetZap70 FoxP3<sup>GFP</sup> mice is set by the tetracycline inducible transgene. The Zap70 level set by the transgene has been shown to be higher than that normally observed in WT CD4<sup>+</sup> CD8<sup>+</sup> DPs but lower than that observed in mature CD4<sup>+</sup> SPs (Saini et al. 2010). It is therefore conceivable that lower Zap70 levels are leading to sub-optimal TCR signalling. This could lead to a failure to undergo complete differentiation and a developmental dead end. However the T<sub>reg</sub> pool in our constitutively dox fed mice appear completely normal, indicating no defects in T<sub>reg</sub> numbers or phenotype. The CD25<sup>Low</sup> FoxP3<sup>+</sup> T-cells observed in our TetZap70 FoxP3<sup>GFP</sup> mice also have normal expression of characteristic T<sub>reg</sub> surface markers including CD5 a good indicator of TCR signalling strength, making failed development unlikely (Fig 1.6). We also show CD25<sup>High</sup> T<sub>reg</sub> developing from CD25<sup>Low</sup> precursors in sorted wt FoxP3<sup>GFP</sup> mice in which Zap70 is under endogenous control (Fig 1.10).

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Although T<sub>reg</sub> development in neonatal mice is dominated by the emergence of a CD25<sup>High</sup> FoxP3<sup>+</sup> population there are still significant percentages of CD25<sup>Low</sup> T<sub>reg</sub> especially at the early time points. Large parts of our data appear to support a purely linear model for T<sub>reg</sub> development, however it is still hard to reconcile this with the observation that CD25<sup>High</sup> thymocytes are more likely to express FoxP3 than CD25<sup>Low</sup> thymocytes, perhaps suggesting a role for both routes during development.

Our study presents evidence both for and against CD25<sup>Low</sup> FoxP3<sup>+</sup> and CD25<sup>High</sup> FoxP3<sup>-</sup> cells acting as precursors to FoxP3<sup>+</sup> CD25<sup>High</sup> T<sub>reg</sub>. It therefore remains possible that both populations act as precursors. Evidence that both routes of development can occur is apparent in our TetZap70 FoxP3<sup>GFP</sup> time course data. When gating on GITR<sup>High</sup> precursors around 90% are CD25<sup>Low</sup> while the remaining 10% are CD25<sup>High</sup> (Fig 1.8). GFP<sup>+</sup> cells appear to be differentiating from both populations, with most cells originating from the more abundant CD25<sup>Low</sup> population. However due to the relatively small percentage of CD25<sup>High</sup> cells; the few GFP<sup>+</sup> cells which do develop suggest an enrichment of precursors in this population. This goes some way to explaining the adoptive transfer experiments used in the literature to describe the 2 step model. Sorting any set number of either population before adoptive transfer would lead to the conclusion that the CD25<sup>High</sup> population is enriched for precursors, this is probably the case; however the bulk of development appears to be going via the CD25<sup>Low</sup> population due to the much higher percentage of these cells. If this is in fact the case in casts doubt over IL-2s role in FoxP3 induction. Perhaps

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suggesting more of a correlative relationship between CD25 and FoxP3 expression rather than an instructive role. This could indicate a role for TCR/co-stimulatory signalling in FoxP3 induction followed by a role for IL-2 in homeostasis when T<sub>reg</sub> mature and egress the thymus.

We observed delayed thymic egress of T<sub>reg</sub> compared to non-regulatory CD4<sup>+</sup> SPs. We also saw egress of both CD25<sup>High</sup> and CD25<sup>Low</sup> populations suggesting CD25<sup>Low</sup> T<sub>reg</sub> can mature and express the necessary markers to egress the thymus in the absence of CD25. It still however remains uncertain if these CD25<sup>Low</sup> cells have undergone a full commitment to the T<sub>reg</sub> lineage or remain unstable and vulnerable to losing FoxP3 expression and becoming 'ex'-T<sub>regs</sub>. Studies using T<sub>reg</sub> fate markers have shown that IL-2 blockade leads to decreased percentages of T<sub>reg</sub> as well as an increase in the percentage of T<sub>reg</sub> which have stopped expressing FoxP3 (exT<sub>reg</sub>) (Rubtsov et al. 2010). The CD25 up regulation on CD25<sup>Low</sup> FoxP3<sup>+</sup> cells, which according to our in vitro studies is dependant on IL-2; could therefore be necessary to maintain a stable T<sub>reg</sub> pool. Previous studies have also observed that output of T<sub>reg</sub> from the thymus is delayed during ontogeny and that development of autoimmune disease in neonatally thymectomised (nTx) mice is due to the escape of self-reactive T-cells without T<sub>reg</sub> to maintain tolerance (Fontenot et al. 2005). Neonatal nTx at 4 days results in T-cell mediated lesions which could be alleviated through the transfer of thymocytes or splenocytes from adult mice (Bonomo et al., 1995.; Life, 1996; Sakaguchi, 1982). Our study confirms this developmental delay also exists in the adult thymus.

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Our results show that after egress into the periphery  $T_{reg}$  can recirculate back into the thymus (Fig 1.7). We show a significant percentage of the thymic  $T_{reg}$  pool are not de novo generated but in fact recirculants. During our experiments adoptively transferring  $CD45.1^+$  T-cells into TetZap70 FoxP3<sup>GFP</sup> chimeras, we see donor cells making up approximately 30% of the thymic  $T_{reg}$  compartment, despite only making up 6% of the total  $CD4^+$  SPs. Further supporting this, constitutively dox fed mice which are then taken off dox for 4 weeks, still contain thymic  $T_{reg}$ , even in the complete absence of TCR signalling and positive selection which has been shown to be crucial for their de novo development (Jordan et al. 2001). Interestingly these  $T_{reg}$  also express the transcription factor helios, which is said to be a marker of thymic derived n $T_{reg}$  (A. M. Thornton et al. 2010). However during the adoptive transfer experiments T-cells were injected into *Rag1*<sup>-/-</sup> hosts which lack a T-cell compartment. The donor cells were therefore most likely undergoing lymphopenia induced proliferation, meaning that although our studies can conclude the presence of recirculating  $T_{reg}$  population it is hard to quantify the relative abundance of these compared to de novo generated  $T_{reg}$ . Previous studies have attempted to quantify the abundance of recirculating T-cell populations in the thymus using *Rag2*<sup>GFP</sup> reporter mice (McCaughy et al. 2007). As *Rag2* expression is tightly regulated in developing thymocytes it is no longer expressed in the periphery, *Rag2*<sup>GFP</sup> T-cells therefore lose GFP expression after thymic egress. The  $T_{reg}$  compartment in these mice contained approximately 60% GFP<sup>-</sup>  $T_{reg}$ . Although these could be resident  $T_{reg}$  which remain in the thymus, taken together with our data it seems likely these are recirculants from the periphery. Studying  $T_{reg}$  development in

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our TetZap70 system therefore offers a distinct advantage compared to other models. Many of the previous models have been developed in mice where the T-cell compartment is under homeostatic control. A major advantage of the TetZap70 system is that we are able to look at de novo T<sub>reg</sub> development in the absence of inference from recirculating populations of cells.

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## Chapter 4

# TCR and cytokine requirement for regulatory T-cell development

### Introduction

The forkhead transcription factor FoxP3 binds to and regulates hundreds of target genes associated with the  $T_{reg}$  lineage. Expression of these genes shapes the  $T_{reg}$  transcriptional landscape, defining the lineage and allowing suppression of immune responses (Hill et al. 2007). However the internal and external cues leading to FoxP3 expression remain poorly understood. FoxP3 expression is thought to occur relatively late during thymic selection, in thymocytes which receive a strong TCR selection signal. Retroviral transfer of  $T_{reg}$  or naïve T-cell  $TCR\alpha$  libraries into  $RAG^{-/-}$  TCR transgenic T-cells shows increased self reactivity of TCRs derived from  $T_{reg}$ .  $T_{reg}$  derived TCRs were more able to induce expansion and auto-immunity upon transfer into lymphopenic hosts but only able mount weak in vitro responses to syngenic APCs, when compared to the reaction to 'foreign' peptide recognised by their transgenic TCRs. Therefore although  $T_{reg}$  bear TCR able to recognise self-antigens, the strength of this TCR signal is weaker than that of conventional T-cells recognising foreign antigen in immune responses (C.Hsieh et al. 2004). The overlap in TCR sequence between  $T_{reg}$  and non- $T_{reg}$  is also reported to be

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very small, suggesting a TCR instructive model for T<sub>reg</sub> development in which self-reactive thymocytes are selected on self-antigens during selection in the thymus.

Co-stimulatory signalling has also been shown to have a cell intrinsic role in the generation of regulatory T-cells (Tai et al. 2005). CD28<sup>-/-</sup> and CD80/86<sup>-/-</sup> mice show large decreases in T<sub>reg</sub> frequency (Lenschow et al. 2000). The Ick binding domain of the CD28 cytoplasmic tail has been shown to be critical for FoxP3 expression (Tai et al. 2005), suggesting co-ordinated TCR and CD28 signalling play a key role in T<sub>reg</sub> development. Several transcription factors such as NFAT and AP-1 are downstream of TCR and CD28 signalling. These have been implicated in T<sub>reg</sub> differentiation and bind to the FoxP3 promoter (Cells et al. 2006). CD40L co-stimulation is also suggested to play a role in thymic T<sub>reg</sub> development. Deficiency in CD40 or CD154 (CD40L) reduces thymic and peripheral T<sub>reg</sub> frequency by approximately 50% (Spence & Green 2008). There is disagreement however over whether this decrease is due to reduced thymic output or in fact due to homeostatic effects in the periphery. More recently it has been suggested that abrogation of CD40-CD154 signalling impeded the homeostasis of thymic resident T<sub>reg</sub> by altering IL-2 levels. It therefore remains a possibility that CD40-CD40L signalling plays an important role in homeostasis rather than thymic T<sub>reg</sub> development.

The TGFβ-smad pathway is thought to induce FoxP3 expression in naïve CD4<sup>+</sup> SPs, generating iT<sub>regs</sub>. However its role in Thymic T<sub>reg</sub> development still remains contentious. Numerous studies have reported severe inflammation of mice with

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complete or T-cell specific deletions of individual components of the TGF $\beta$  signalling pathway (M. O. Li et al. 2006; Gorelik & R. A. Flavell 2000)(Y. Liu et al. 2008; Lucas et al. 2000). However no consensus has been reached as to the affect TGF $\beta$  has on thymic T<sub>reg</sub> development. One recent study generated mice in which the smad binding site within the FoxP3 locus was removed. This showed TGF $\beta$  was dispensable for T<sub>reg</sub> development in adult and new born mice, while demonstrating a key requirement for TGF $\beta$ -smad signalling for T<sub>reg</sub> generation in the gut (Schlenger et al. 2012).

IL-2/IL-2R signalling is essential for thymic T<sub>reg</sub> development and peripheral homeostasis(Malek 2008). Mice lacking IL-2 or IL2R $\alpha$  (CD25) exhibit an approximate 50% decrease in T<sub>reg</sub> frequencies (Fontenot, Rasmussen, Gavin & A. Y. Rudensky 2005). IL-2<sup>-/-</sup>/CD25<sup>-/-</sup> mice undergo very severe systemic auto-immunity paralleling that seen in FoxP3<sup>-/-</sup> mice (Fontenot et al. 2003). The T-cells within these mice undergo uncontrolled hyper activation and animals succumb to lethal auto-immunity within 4-12 weeks of age (Schorle et al. 1991). Restoring the T<sub>reg</sub> compartment is sufficient to prevent auto-immunity, suggesting defective T<sub>regs</sub> are responsible (Malek et al. 2002). One difference between FoxP3<sup>-/-</sup> mice and IL-2<sup>-/-</sup>/CD25<sup>-/-</sup> mice is the latter still contain approximately 50% of their T<sub>regs</sub>. These T<sub>reg</sub> essentially lack all CD25 expression and are found in very low proportions within peripheral lymphoid organs (Bayer et al. 2007). CD25<sup>lo</sup> FoxP3<sup>+</sup> T<sub>reg</sub> are still able to suppress immune responses and inhibit T-cell proliferation but are unable to proliferate efficiently themselves, accounting for the slower pace of auto-immunity within



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the IL-2<sup>-/-</sup> mice. IL-2 is described in the 2 step model as initiating thymic FoxP3 expression via STAT5 (Chan-Wang Joaquim Lio & C.-S. Hsieh 2008). Conditional STAT5 deletion leads to a drastic reduction in FoxP3<sup>+</sup> cells (M. A. Burchill et al. 2007). Introduction of a Bcl-2 transgene leads to T<sub>reg</sub> development in the absence of STAT5 signalling (Josefowicz & A. Rudensky 2009). Suggesting T<sub>reg</sub> require an IL-2 dependant survival signal during homeostasis, and may not solely rely on IL-2 to induce FoxP3 expression. IL15R-IL15 signalling is also capable of activating STAT5 signalling. Even though IL-2 and IL-15 share common receptor subunits, CD122 (IL2Rβ) and γc, IL15Rα<sup>-/-</sup> mice are autoimmune free and contain normal numbers of FoxP3<sup>+</sup> T<sub>reg</sub>. It is therefore likely that redundancy between the γc family cytokines during de novo T<sub>reg</sub> development exists (M. A. Burchill et al. 2007).

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## Results

### CD80/86 blockade reduces T<sub>reg</sub> development

Co-stimulatory signalling is thought to play a crucial role in T<sub>reg</sub> differentiation, as frequencies of T<sub>reg</sub> are reduced in CD28<sup>-/-</sup> and CD80/86<sup>-/-</sup> mice (Lenschow et al. 2000). We therefore investigated if CD28 co-stimulation plays an important role in de novo T<sub>reg</sub> differentiation in TetZap70 FoxP3<sup>GFP</sup> chimeras. To achieve this we took advantage of CD80 and CD86 blocking antibodies, 16-10A1 and GL-1. TetZap70 FoxP3<sup>GFP</sup> chimeras received a 1mg dose of a CD80/86 blocking antibody via an ip injection every 2 days beginning on day-1 before being fed dox. Their thymus was analysed by FACs 6 days after commencing dox feeding. Bl/6 mice which received the CD80/86 blockade treatment for 6 days as controls, showed a 5-fold decrease in thymic T<sub>reg</sub> (Fig 2.1A). While CD80/86 blockade had no effect on CD4<sup>+</sup> SP percentages in the chimeras, we did observe a significant reduction in the frequency of GFP<sup>+</sup> T<sub>reg</sub> (Fig 2.1 B&C). Interestingly the T<sub>reg</sub> compartment in the chimeras which received the CD80/86 blockade expressed low levels of CD25 while PBS controls contained both CD25<sup>Hi</sup> and CD25<sup>Lo</sup> GFP<sup>+</sup> populations. The reduction in T<sub>reg</sub> frequencies reported in CD28<sup>-/-</sup> mice were similarly observed in Bl/6 mice which received the antibody blockade. This suggests blocking antibodies successfully locate to the thymus and block CD28 co-stimulation. The observations that this blockade dramatically reduces thymic T<sub>reg</sub> in TetZap70 FoxP3<sup>GFP</sup> chimeras confirm a crucial role for signalling elicited by CD80/86 in de novo T<sub>reg</sub> development.

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### **CD40-CD40L signalling plays no role in thymic de novo T<sub>reg</sub> development**

Next we wished to investigate the roles of additional forms of co-stimulatory signalling on T<sub>reg</sub> development. It has been reported that during thymic selection CD40L is specifically up-regulated on FoxP3<sup>+</sup> thymic T<sub>reg</sub> where it induces clonal expansion within the medulla (Spence & Green 2008). We therefore investigated the affect of blocking CD40L on de novo T<sub>reg</sub> development. TetZap70 FoxP3<sup>GFP</sup> chimeras received 0.5mg of a CD40L blocking antibody MR1 one day prior to being fed dox. The chimeras received the blocking antibody treatment every 2 days before their thymus was analysed by FACs. Bl/6 control mice which received similar CD40L blocking antibody treatment exhibited a ≈50% decrease in thymic T<sub>reg</sub> (Fig 2.2A). In TetZap70 FoxP3<sup>GFP</sup> chimeras however, treatment with the CD40L blocking antibody had no effect on the T<sub>reg</sub> compartment. Thymic T<sub>reg</sub> percentages in these mice were identical to PBS injected controls and consistent with historical pooled time course data (Fig 2.2 B&C) Taken together these results indicate no role for CD40L in development of T<sub>reg</sub> in the thymus, perhaps instead suggesting a homeostatic role for mature T<sub>reg</sub> which recirculate back into the thymus from the periphery.

### **A short window of TCR signalling is sufficient to induce FoxP3 expression**

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TCR signalling is thought to be crucial for  $T_{reg}$  differentiation. In TCR transgenic mice,  $T_{reg}$  only develop when cognate antigen is present (Jordan et al. 2001). However it remains unclear when TCR signalling is required during thymic development of  $T_{reg}$ . To investigate this, we studied  $T_{reg}$  development in TetZap70FoxP3<sup>GFP</sup> chimeras in which Zap70 expression is restricted to the early stages of selection. It has been shown previously that an ip injection of 2mg methacycline induces Zap70 expression for approximately 48 hours after which it is rapidly lost (Sinclair et al. 2011). TetZap70FoxP3<sup>GFP</sup> mice were therefore injected with 2mg methacycline (ip) and their thymus analysed by FACs. CD4<sup>+</sup> SP thymocytes from Methacycline pulsed TetZap70FoxP3<sup>GFP</sup> mice expressed low levels of Zap70 4 days after injection, and Zap70 levels dropped further by day 6. CD5 attenuates TCR signalling through recruitment of the tyrosine phosphatase SHP-1 (Azzam et al. 2001). CD5 expression on thymocytes is therefore proportional to the strength of the TCR signal strength cells are exposed to. CD5 expression levels on methacycline pulsed TetZap70 FoxP3<sup>GFP</sup> thymocytes were reduced compared to Bl/6 controls (Fig 2.3C). Surprisingly  $T_{reg}$  frequency's as a percentage of CD4<sup>+</sup> SPs were identical in both the methacycline pulsed and dox fed controls (Fig 2.3B). As a percentage of total thymocytes, methacycline and dox mice appear to have similar percentages at day 4 but  $T_{reg}$  percentage dropped slightly by day 6 in the methacycline pulsed mice. In methacycline pulsed mice CD4<sup>+</sup> SP percentages drop after day 4, therefore the increase in  $T_{reg}$  as a percentage of CD4<sup>+</sup> SPs may reflect increased survival of  $T_{reg}$  in the absence of TCR signalling. This was reflected by the maintenance of  $T_{reg}$  frequency as a percentage of total thymus

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on day 4. The drop in percentage at day 6 can be explained by a drop in CD4<sup>+</sup> SP numbers most likely due to cell death in the absence of a TCR induced survival signal. This data suggests that an early TCR signal is sufficient to cause FoxP3 expression and that FoxP3 expression maintained in the absence of a TCR signal.

### **TGFβ blockade has no affect on de novo T<sub>reg</sub> development.**

Since an early TCR signal was sufficient for FoxP3 induction next we aimed to investigate if the later stages of development were reliant on cytokine signalling. TGFβ is heavily implicated in causing naïve CD4<sup>+</sup> SPs to express FoxP3 in the periphery, known as iT<sub>regs</sub>. However its role in FoxP3 induction in the thymus remains more contentious. To investigate the role TGFβ plays in the thymic induction of FoxP3, TetZap70 FoxP3<sup>GFP</sup> chimeras were fed dox and treated with 1mg of a TGFβ blocking antibody every 2 days. The first antibody treatment was administered 1 day prior to dox feeding. The thymus was then analysed on days 4 and 6 by FACs. The frequency of GFP<sup>+</sup> cells within the CD4<sup>+</sup> SP compartment in the mice treated with TGFβ mAb appeared similar to controls both at d4 and d6 after dox feeding (Fig 2.4 A&B). This data suggests that there is no role for TGFβ in thymic de novo T<sub>reg</sub> development. However, since we observed no phenotype in Bl/6 mice which received the TGFβ mAb, it remains possible that the blocking antibody treatment did not completely abrogate TGFβ signalling.

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## **IL-2 is critical to the development of CD4<sup>+</sup> SP CD25<sup>Hi</sup> FoxP3<sup>+</sup> T<sub>reg</sub>**

Next we wished to analyse the role IL-2 plays during de novo T<sub>reg</sub> development. IL-2 is thought to be crucial for both FoxP3 induction and T<sub>reg</sub> homeostasis (Chan-Wang Joaquim Lio & C.-S. Hsieh 2008)(Setoguchi et al. 2005). The requirement for IL-2 during de novo T<sub>reg</sub> development has not been directly analysed previously. To determine the effects of IL-2 neutralisation on the developing T<sub>reg</sub>, TetZap70 FoxP3<sup>GFP</sup> chimeras and WT controls received a 1mg dose of IL-2 neutralising antibodies every 2 days starting one day prior to dox feeding. The thymocytes from these mice were then analysed by FACs at d4 and d6. Bl/6 control mice which received the IL-2-neutralising antibody treatment for 6 days showed a 50% decrease in thymic CD4<sup>+</sup> SP CD25<sup>High</sup> CD44<sup>Int</sup> T<sub>reg</sub>, consistent with observations previously made in IL-2<sup>-/-</sup> mice (Fontenot, Rasmussen, Gavin & A. Y. Rudensky 2005). The frequency of GFP<sup>+</sup> cells was not affected by IL-2 blockade when compared to controls (Fig 2.5 A&B). However chimeras which received the IL-2 blocking antibody specifically lacked GFP<sup>+</sup> CD25<sup>Hi</sup> cells compared to PBS treated controls,(Fig 2.5 A&C). Although the size of the total GFP<sup>+</sup> compartment in IL-2 treated mice was not statistically different from that of controls there was a trend towards their having fewer GFP<sup>+</sup> cells (Fig 2.5D). These data could suggest that CD25 up-regulation on developing T<sub>reg</sub> is IL-2 dependant and these develop from a CD25<sup>Low</sup> GFP<sup>+</sup> precursor. It is also consistent with 2 routes of T<sub>reg</sub> development, one via a CD25<sup>Hi</sup> GFP<sup>-</sup> precursor, and one via a CD25<sup>Low</sup> FoxP3<sup>+</sup> precursor where

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development is IL-2 independent. It is possible that the lack of a prominent CD4<sup>+</sup> SP CD25<sup>High</sup> GFP<sup>+</sup> compartment in TetZap70 FoxP3<sup>GFP</sup> chimeras is due to the lack of the correct cytokine milieu during selection in the thymus.

We have shown that blocking IL-2 stopped generation of CD4<sup>+</sup> SP CD25<sup>High</sup> GFP<sup>+</sup> cells. Next we asked if exogenous IL-2 was capable of enhancing development of CD4<sup>+</sup> SP CD25<sup>High</sup> GFP<sup>+</sup> T<sub>reg</sub> cells. IL-2 monoclonal antibodies when coupled to recombinant IL-2 have been shown to increase the biological half-life of IL-2 (Boyman et al. 2006). Therefore TetZap70 FoxP3<sup>GFP</sup> chimeras were fed dox food and injected with IL-2-antibody immune complexes every 2 days, beginning one day prior to dox feeding. When compared to controls, IL-2 treated chimeras showed increased CD25 expression (Fig 2.6A). This also corresponded with an increase in absolute T<sub>reg</sub> cell numbers on days 4 and 6 in the IL-2 treated mice (Fig 2.6B). Taken together this data would suggest that an exogenous source of IL-2, is capable of both increasing CD25 expression levels and causing either proliferation or increased differentiation of T<sub>reg</sub>.

### **IL-15 plays an important role in CD4<sup>+</sup> GFP<sup>+</sup> CD25<sup>low</sup> T<sub>reg</sub> development**

Next we wished to investigate a role for IL-15 in T<sub>reg</sub> homeostasis. It has been reported that IL-15 plays a relatively minor role in T<sub>reg</sub> maintenance and development as IL-2R $\alpha$ <sup>-/-</sup> and IL-2R $\beta$ <sup>-/-</sup> mice have FoxP3<sup>+</sup> populations of comparable size. However it remains unclear as to the extent of the redundancy that exists between IL-15 and IL-2 in T<sub>reg</sub> differentiation and homeostasis (M. A.

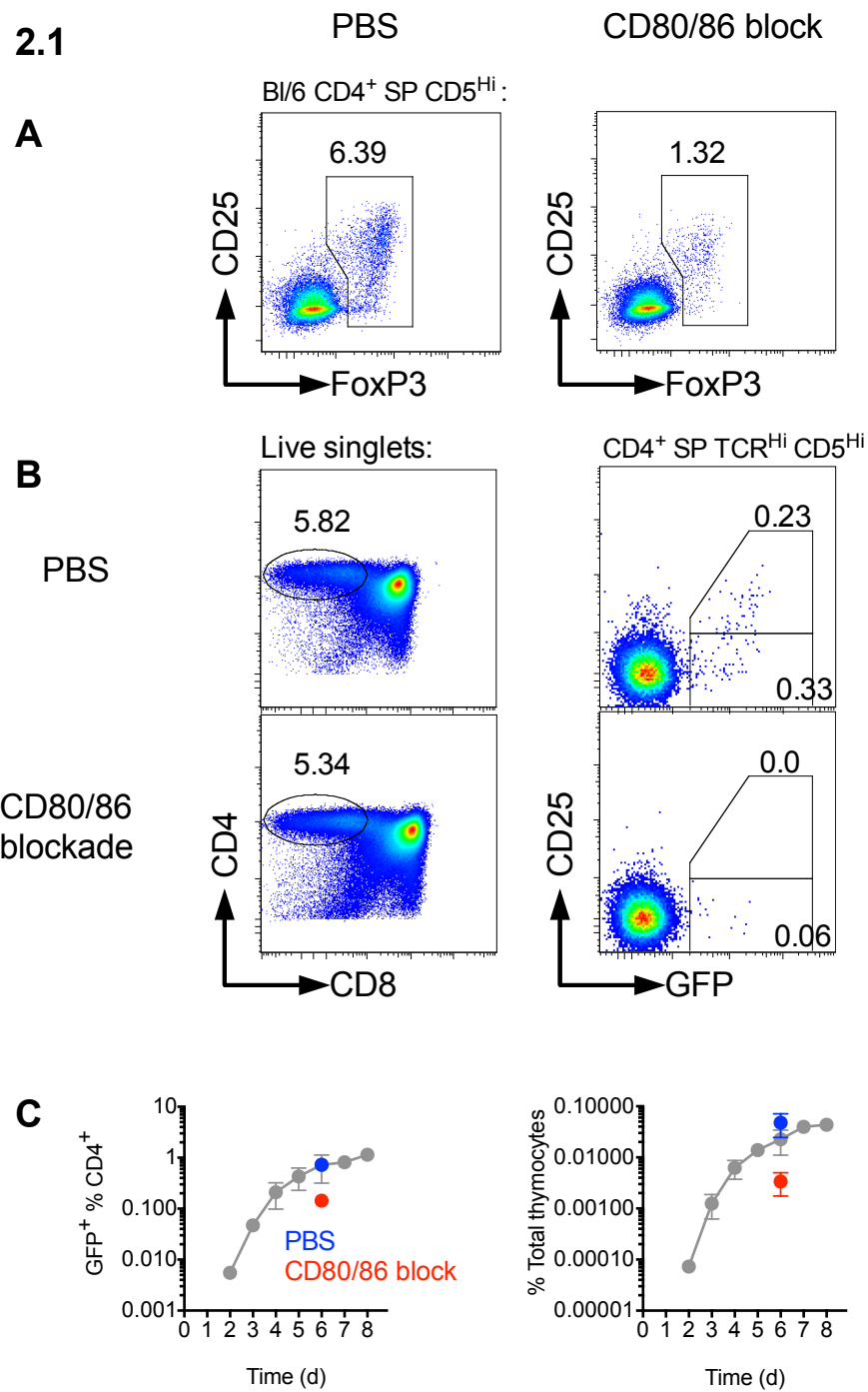
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Burchill et al. 2007). We therefore aimed to further investigate the role for IL-15 in T<sub>reg</sub> biology. In order to achieve this we sub lethally irradiated Rag1<sup>-/-</sup> IL-15Rα<sup>-/-</sup> host mice and injected them with 5x10<sup>6</sup> congenically labelled B1/6 bone marrow cells. These were left for 6 weeks to reconstitute before their thymus and LNs were analysed by FACs. The numbers of CD4<sup>+</sup> SP cells in the thymus and LNs of Rag1<sup>-/-</sup> IL-15Rα<sup>-/-</sup> host chimeras were comparable to numbers in Rag1<sup>-/-</sup> host controls chimeras (Fig 2.7B). Rag1<sup>-/-</sup> IL-15Rα<sup>-/-</sup> chimeras contained similar percentages of thymic FoxP3<sup>+</sup> T<sub>reg</sub> as Rag1<sup>-/-</sup> hosts, but showed an approximate 50% reduction of CD4<sup>+</sup> SP FoxP3<sup>+</sup> cells in the periphery (Fig 2.7 A&B). This suggests a redundant role for IL-15 and IL-2 in the thymus and points towards a homeostatic role for IL-15 in maintenance of T<sub>reg</sub> in the periphery.

We next decided to look more closely at the roles IL-15 and IL-2 play in de novo T<sub>reg</sub> development in the thymus. To investigate this we used IL-15R<sup>-/-</sup> Rag1<sup>-/-</sup> mice as hosts for TetZap70 FoxP3<sup>GFP</sup> chimeras and treated them with or without IL-2 blocking antibody whilst feeding them dox. Analysing the thymus of IL-15R<sup>-/-</sup> Rag1<sup>-/-</sup> TetZap70 FoxP3<sup>GFP</sup> chimeras revealed a significant reduction in the frequency of GFP<sup>+</sup> cells compared to Rag1<sup>-/-</sup> control chimeras (Fig 2.8 C). IL-2 blockade prevented development of CD4<sup>+</sup> SP CD25<sup>Hi</sup> GFP<sup>+</sup> cells in both chimeras (Fig 2.8 A&C). In contrast development of the CD4<sup>+</sup> SP CD25<sup>Low</sup> GFP<sup>+</sup> was unaffected (Fig 2.8C). These data indicate a requirement for both IL-2 and IL-15 for efficient CD4<sup>+</sup> SP CD25<sup>High</sup> GFP<sup>+</sup> T<sub>reg</sub> development, while CD4<sup>+</sup> SP CD25<sup>Low</sup> GFP<sup>+</sup> cells rely solely on IL-15.



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**Figure 2.1 CD80/86 antibody blockade reduces Thymic T<sub>reg</sub> frequencies.**

Rag1<sup>-/-</sup> mice were irradiated and injected with 5M TetZap70 FoxP3<sup>GFP</sup> bone marrow cells iv. Bone marrow was harvested as described in materials and methods. Chimeras were left for 6 weeks to reconstitute before being treated along with Bl/6 controls with 0.5mg of a CD80 and 0.5mg of a CD86 blocking antibody via a single ip injection (16-10A1, GL-1). Alternatively both chimeras and Bl/6 mice were treated with PBS alone. The following day chimeras were fed dox, receiving additional doses of blocking antibody or PBS every 2 days until day 6 when the thymus was analysed by FACs. **A:** FACs plots comparing CD25 and FoxP3 expression on Bl/6 mice treated with PBS (left) or CD80/86 blockade (right). **B:** FACs plots comparing CD4 and CD8 expression on live singlets (left panel) in PBS treated (top) or CD80/86 blocked mice (bottom). CD25 and GFP expression was then compared on TCR<sup>Hi</sup> CD5<sup>Hi</sup> CD4<sup>+</sup> SP cells (right panel) for PBS treated (top) and CD80/86 blocked cells (bottom). **C:** Line graphs comparing TCR<sup>Hi</sup> CD5<sup>Hi</sup> CD4<sup>+</sup> SP GFP<sup>+</sup> frequency in PBS treated (blue) and CD80/86 blocked (red) mice to historical TetZap70 FoxP3<sup>GFP</sup> chimera time course data (grey). Expressed as a percentage of TCR<sup>Hi</sup> CD5<sup>Hi</sup> CD4<sup>+</sup> SPs (left) and total thymocytes (right).

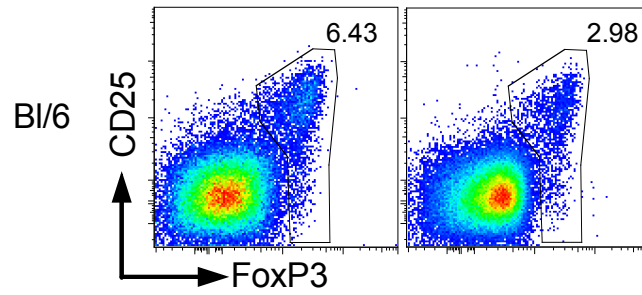
2.2

PBS

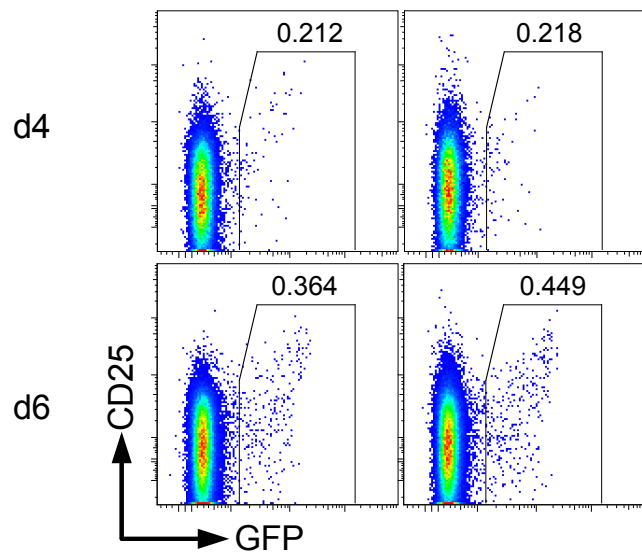
CD40L block

CD4<sup>+</sup> SP TCR<sup>High</sup> CD5<sup>High</sup>:

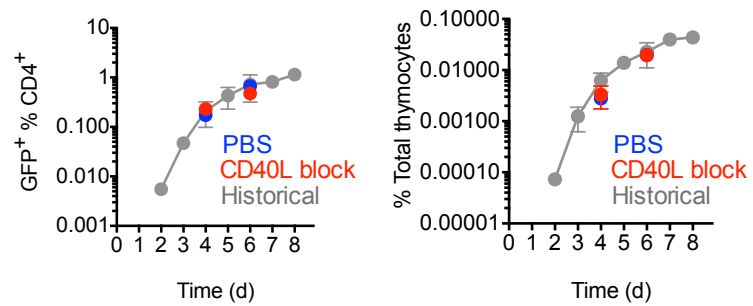
A



B



C



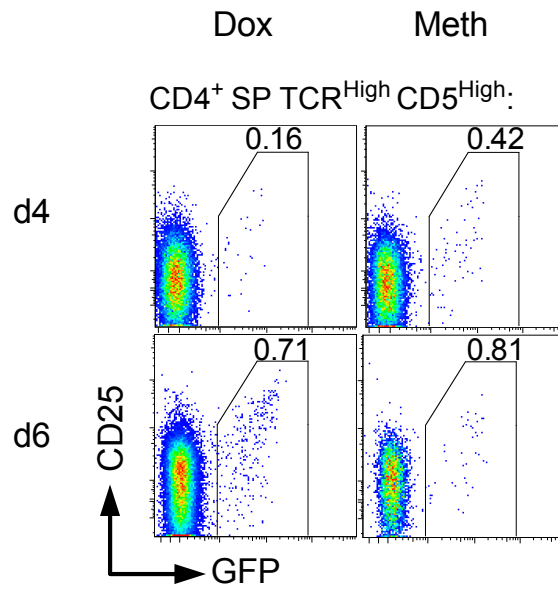
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**Figure 2.2 CD40L blockade has no affect de novo T<sub>reg</sub> development.**

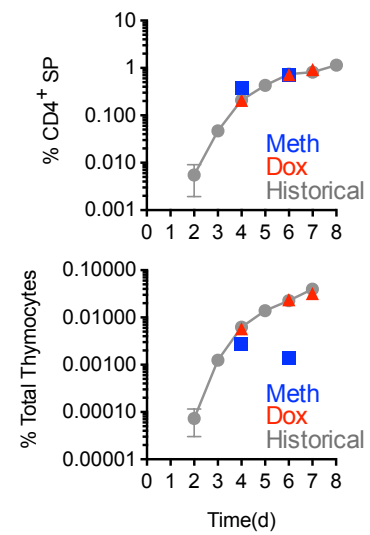
Rag1<sup>-/-</sup> mice were irradiated and injected with 5M TetZap70 FoxP3<sup>GFP</sup> bone marrow cells iv. Bone marrow was harvested as described in materials and methods. Chimeras were left for 6 weeks to reconstitute before being treated along with Bl/6 controls with 0.5mg of a CD40L blocking antibody via a single ip injection (MR1). Alternatively both chimeras and Bl/6 mice were treated with PBS alone. The following day chimeras were fed dox, receiving additional doses of blocking antibody or PBS every 2 days until day 4 or 6 when the thymus was analysed by FACs. **A:** FACs plots comparing CD25 and FoxP3 expression on Bl/6 mice treated with PBS (left) or CD40L blockade (right). **B:** FACs plots comparing CD25 and GFP expression on TCR<sup>Hi</sup> CD5<sup>Hi</sup> CD4<sup>+</sup> SP thymocytes from PBS treated (left) and CD40L blocked cells (right) on days 4 (top panel) and 6 (bottom panel). **C:** Line graphs comparing TCR<sup>Hi</sup> CD5<sup>Hi</sup> CD4<sup>+</sup> SP GFP<sup>+</sup> frequency in PBS treated (blue) and CD40L blocked (red) mice to historical TetZap70 FoxP3<sup>GFP</sup> chimera time course data (grey). Expressed as a percentage of TCR<sup>Hi</sup> CD5<sup>Hi</sup> CD4<sup>+</sup> SPs (left) and total thymocytes (right).

## 2.3

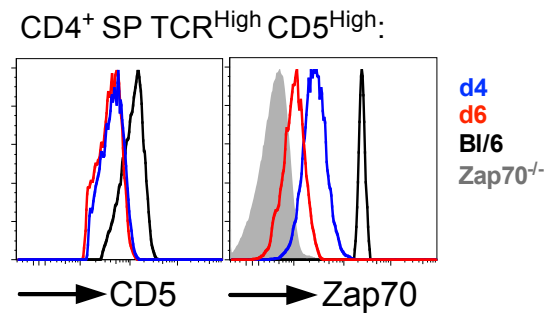
**A**



**B**



**C**



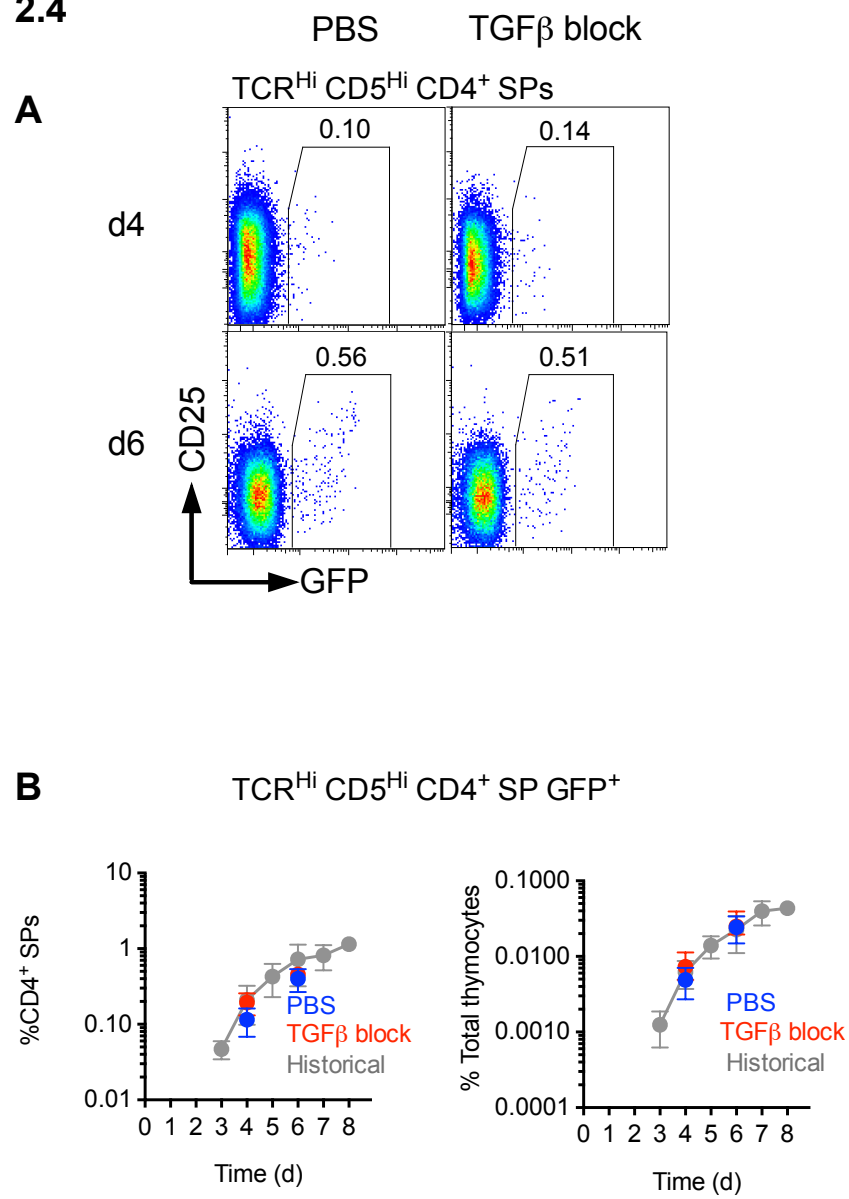
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**Figure 2.3 Early expression of Zap70 supports de novo T<sub>reg</sub> development.**

Rag1<sup>-/-</sup> mice were irradiated and injected with 5M TetZap70 FoxP3<sup>GFP</sup> bone marrow cells iv. Bone marrow was harvested as described in materials and methods. Chimeras were left for 6 weeks to reconstitute before being treated with 2mgs of methacycline via a single ip injection. The thymus of the methacycline pulsed chimeras was then analysed by FACs 4 and 6 days later.

**A:** FACs plots comparing CD25 and GFP expression on TCR<sup>Hi</sup> CD5<sup>Hi</sup> CD4<sup>+</sup> SP thymocytes from dox fed (left) and methacycline pulsed (right) on days 4 (top panel) and 6 (bottom panel). **B:** Line graphs comparing TCR<sup>Hi</sup> CD5<sup>Hi</sup> CD4<sup>+</sup> SP GFP<sup>+</sup> frequency in methacycline pulsed (blue) and dox fed mice (red) to historical TetZap70 FoxP3<sup>GFP</sup> chimera time course data (grey). Expressed as a percentage of TCR<sup>Hi</sup> CD5<sup>Hi</sup> CD4<sup>+</sup> SPs (top) and total thymocytes (bottom). **C:** Histograms comparing CD5 expression (left) and Zap70 expression (right) on TCR<sup>Hi</sup> CD5<sup>Hi</sup> CD4<sup>+</sup> SP thymocytes from methacycline pulsed mice analysed after 4 days (blue) 6 days (red), Bl/6 control mice (black) or Zap70<sup>-/-</sup> mice (grey).

## 2.4



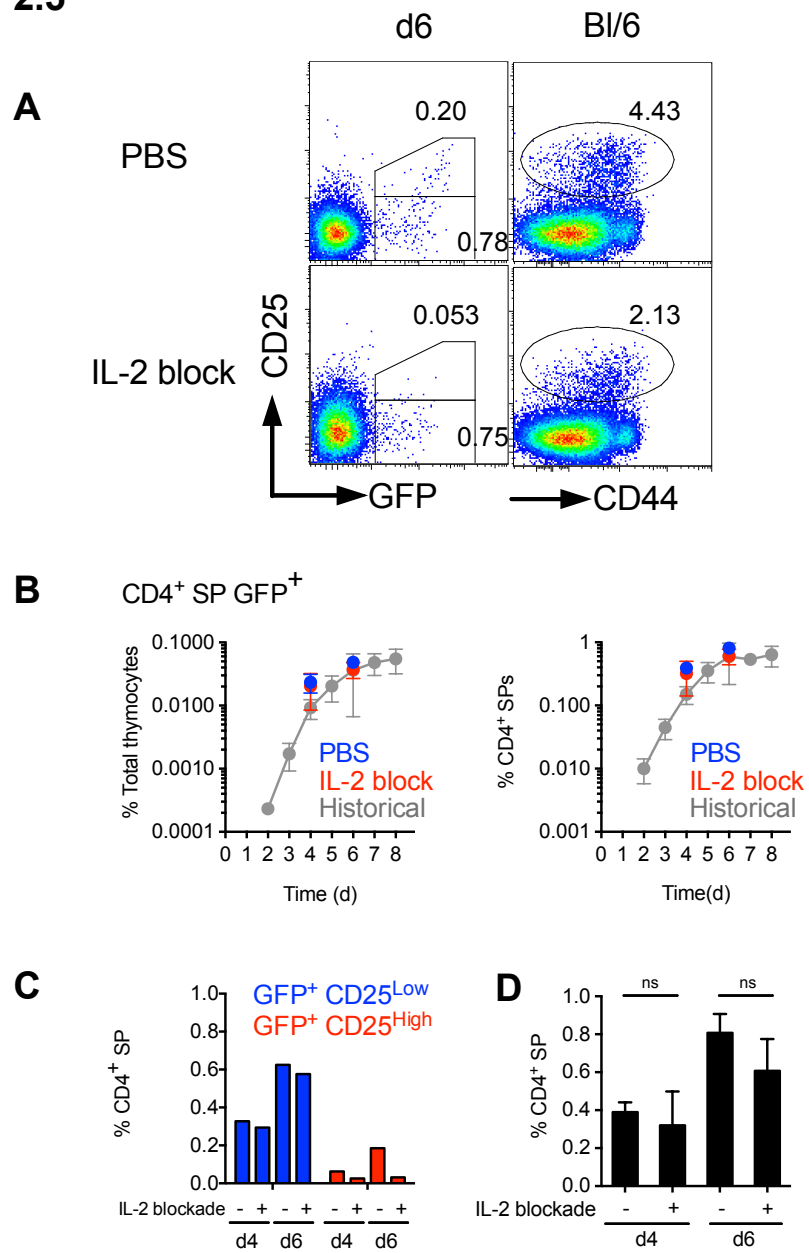


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**Figure 2.4 TGF $\beta$  blockade has no affect on de novo T<sub>reg</sub> development.**

Rag1<sup>-/-</sup> mice were irradiated and injected with 5M TetZap70 FoxP3<sup>GFP</sup> bone marrow cells iv. Bone marrow was harvested as described in materials and methods. Chimeras were left for 6 weeks to reconstitute before being treated with 1mg of a TGF $\beta$  blocking antibody via a single ip injection (1D11.11.8). The following day chimeras were fed dox, receiving additional does of blocking antibody or PBS every 2 days until day 6 when the thymus was analysed by FACs. **A:** FACs plots comparing CD25 and GFP expression on TCR<sup>Hi</sup> CD5<sup>Hi</sup> CD4<sup>+</sup> SP thymocytes from PBS treated (left) and TGF $\beta$  blocked (right) on days 4 (top panel) and 6 (bottom panel). **B:** Line graphs comparing TCR<sup>Hi</sup> CD5<sup>Hi</sup> CD4<sup>+</sup> SP GFP<sup>+</sup> frequency in PBS treated (blue) and TGF $\beta$  blocked mice (red) to historical TetZap70 FoxP3<sup>GFP</sup> chimera time course data (grey). Expressed as a percentage of TCR<sup>Hi</sup> CD5<sup>Hi</sup> CD4<sup>+</sup> SPs (left) and total thymocytes (right).

## 2.5

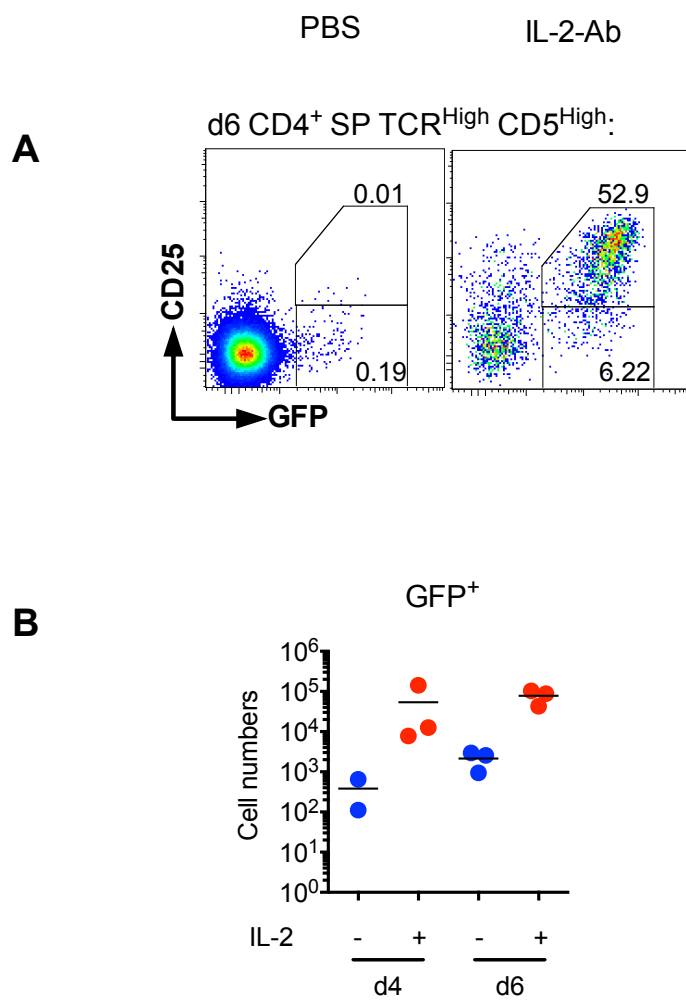


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**Figure 2.5 IL-2 blockade reduced CD25 expression on thymic T<sub>reg</sub>.**

Rag1<sup>-/-</sup> mice were irradiated and injected with 5M TetZap70 FoxP3<sup>GFP</sup> bone marrow cells iv. Bone marrow was harvested as described in materials and methods. Chimeras were left for 6 weeks to reconstitute before being treated with 1mg of an IL-2 blocking antibody along with Bl/6 controls via a single ip injection (0.5mg JES6-5H4, 0.5mg S4B6). The following day chimeras were fed dox, receiving additional doses of blocking antibody or PBS, along with Bl/6 controls, every 2 days until day 6 when the thymus was analysed by FACs. **A:** FACs plots comparing CD25 and GFP expression on d6 TCR<sup>Hi</sup> CD5<sup>Hi</sup> CD4<sup>+</sup> SP thymocytes (left panel) from PBS treated (top) and IL-2 blocked (bottom) chimeras. FACs plots comparing thymic T<sub>reg</sub> (CD25<sup>Hi</sup> CD44<sup>Int</sup>) percentages in Bl/6 mice (right panel) treated with either PBS (top) or IL-2 blocking antibody (bottom). **B:** Line graphs comparing TCR<sup>Hi</sup> CD5<sup>Hi</sup> CD4<sup>+</sup> SP GFP<sup>+</sup> frequency in PBS treated (blue) and IL-2 blocked mice (red) to historical TetZap70 FoxP3<sup>GFP</sup> chimera time course data (grey). Expressed as a percentage of TCR<sup>Hi</sup> CD5<sup>Hi</sup> CD4<sup>+</sup> SPs (right) and total thymocytes (left). **C:** Bar charts showing TCR<sup>Hi</sup> CD5<sup>Hi</sup> CD4<sup>+</sup> SP GFP<sup>+</sup> thymocyte frequency, with or without IL-2 blockade on days 4 and 6 (right panel). Thymocytes have then been further subdivided depending on CD25 expression (left panel) into CD25<sup>High</sup> (red) or CD25<sup>Low</sup> (blue). Frequencies displayed as a percentage of TCR<sup>Hi</sup> CD5<sup>Hi</sup> CD4<sup>+</sup> SPs.

## 2.6



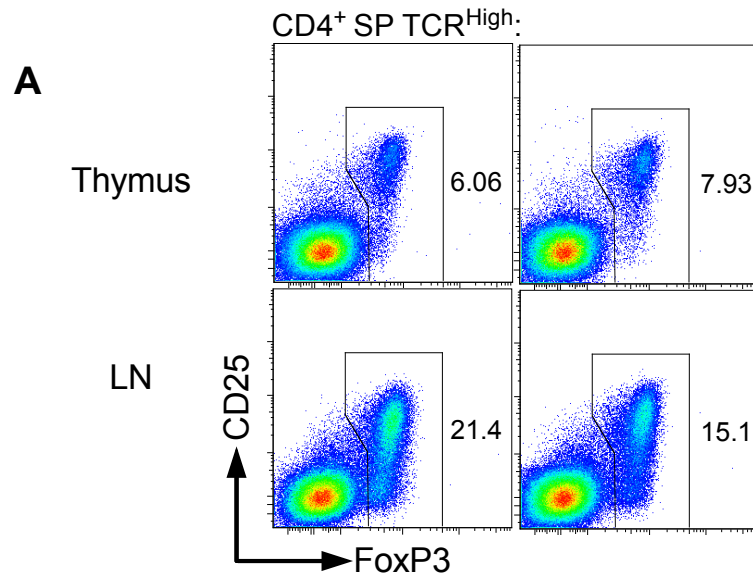
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**Figure 2.6 In vivo IL-2 treatment increases CD25 expression on T<sub>reg</sub>.**

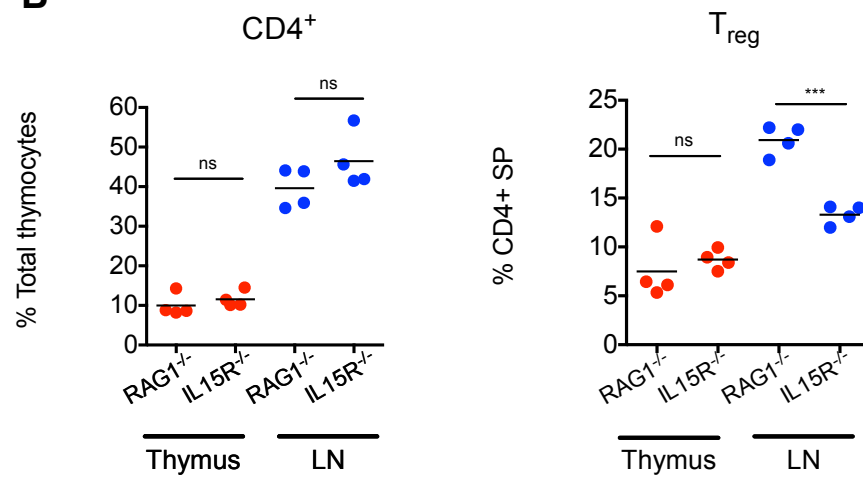
Rag1<sup>-/-</sup> mice were irradiated and injected with 5M TetZap70 FoxP3<sup>GFP</sup> bone marrow cells iv. Bone marrow was harvested as described in materials and methods. Chimeras were left for 6 weeks to reconstitute before being treated with IL-2-Antibody complexes via a single ip injection. 50µg of IL-2 antibody (JES6-1A12) we left on ice with 5µg recombinant IL-2 (peprotech) for 30 mins prior to injection. The following day chimeras were fed dox, receiving additional does of IL-2-antibody or PBS every 2 days until day 6 when the thymus was analysed by FACs. **A:** FACs plots comparing CD25 and GFP expression on d6 TCR<sup>Hi</sup> CD5<sup>Hi</sup> CD4<sup>+</sup> SP thymocytes from PBS treated (left) and IL-2-antibody treated (right) chimeras. **B:** Cell numbers comparing TCR<sup>Hi</sup> CD5<sup>Hi</sup> CD4<sup>+</sup> SP GFP<sup>+</sup> frequency in PBS treated (blue, n=3) and IL-2-antibody treated mice (red, n=3) on days 4 (left) and 6 (right).

2.7

RAG1<sup>-/-</sup> IL15R<sup>-/-</sup> RAG1<sup>-/-</sup>



**B**



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**Figure 2.7 Characterisation of IL-15R<sup>-/-</sup> Rag1<sup>-/-</sup> host B1/6 chimeras**

IL-15R<sup>-/-</sup> Rag1<sup>-/-</sup> and Rag1<sup>-/-</sup> mice were irradiated and injected with 5M B1/6 CD45.1 bone marrow cells iv. Bone marrow was harvested and T-cell depleted using MACs beads, as described in materials and methods. Chimeras were left for 6 weeks to reconstitute before being analysed by FACS **A:** FACS plots comparing CD25 and FoxP3 expression on TCR<sup>Hi</sup> CD4<sup>+</sup> SP thymocytes (top panel) and LN cells (bottom panel) from Rag1<sup>-/-</sup> hosts (left) and IL-15R<sup>-/-</sup> Rag1<sup>-/-</sup> (right) chimeras. **B:** TCR<sup>Hi</sup> CD4<sup>+</sup> SP cell frequencies (left) and TCR<sup>Hi</sup> CD4<sup>+</sup> SP FoxP3<sup>+</sup> cell frequencies (right) in IL-15R<sup>-/-</sup> Rag1<sup>-/-</sup> and Rag1<sup>-/-</sup> chimeras showing thymocytes (red) and n cells (blue).

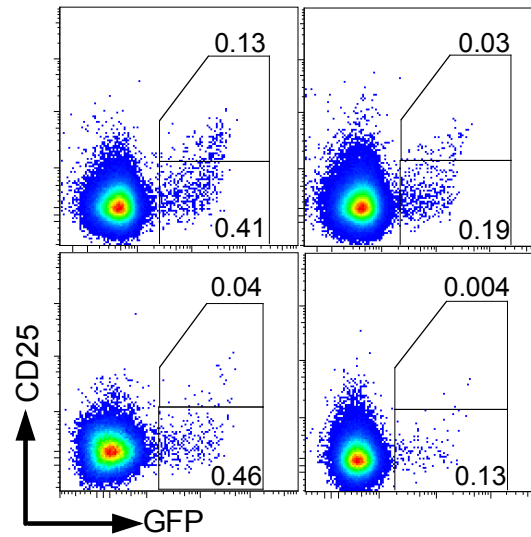
## 2.8

RAG1<sup>-/-</sup> IL-15R<sup>-/-</sup> RAG1<sup>-/-</sup>  
CD4<sup>+</sup> SP TCR<sup>Hi</sup> CD5<sup>Hi</sup>:

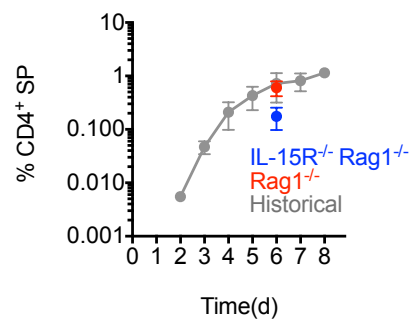
**A**

PBS

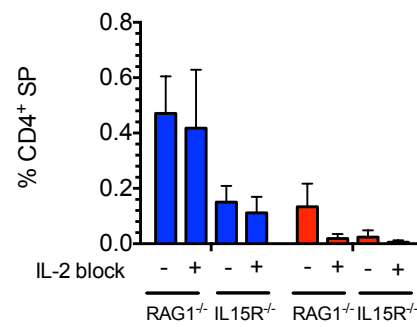
IL-2  
blockade



**B**



**C**





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**Figure 2.8 Reduced T<sub>reg</sub> frequencies in IL-15R<sup>-/-</sup> Rag1<sup>-/-</sup> host chimeras.**

IL-15R<sup>-/-</sup> Rag1<sup>-/-</sup> and Rag1<sup>-/-</sup> mice were irradiated and injected with 5M TetZap70 FoxP3<sup>GFP</sup> bone marrow cells iv. Bone marrow was harvested and injected as described in materials and methods. Chimeras were left for 6 weeks to reconstitute before being treated with 1mg of an IL-2 blocking antibody along via a single ip injection (0.5mg JES6-5H4, 0.5mg S4B6). The following day chimeras were fed dox, receiving additional doses of blocking antibody or PBS, every 2 days until days 4 and 6 when the thymus was analysed by FACs. **A:** FACs plots comparing CD25 and GFP expression on TCR<sup>Hi</sup> CD5<sup>Hi</sup> CD4<sup>+</sup> SP thymocytes treated with either PBS (top panel) or IL-2 blocking antibody (bottom panel) from Rag1<sup>-/-</sup> hosts (left) and IL-15R<sup>-/-</sup> Rag1<sup>-/-</sup> hosts (right). **B:** Line graphs comparing TCR<sup>Hi</sup> CD5<sup>Hi</sup> CD4<sup>+</sup> SP GFP<sup>+</sup> frequency in IL-15R<sup>-/-</sup> Rag1<sup>-/-</sup> hosts (blue) and Rag1<sup>-/-</sup> hosts (red) to historical TetZap70 FoxP3<sup>GFP</sup> chimera time course data (grey). Expressed as a percentage of TCR<sup>Hi</sup> CD5<sup>Hi</sup> CD4<sup>+</sup> SPs. **C:** Bar charts showing TCR<sup>Hi</sup> CD5<sup>Hi</sup> CD4<sup>+</sup> SP GFP<sup>+</sup> thymocyte frequency, with or without IL-2 blockade in IL-15R<sup>-/-</sup> Rag1<sup>-/-</sup> or Rag1<sup>-/-</sup> host chimeras. Thymocytes have then been further subdivided depending on CD25 expression into CD25<sup>High</sup> (red) or CD25<sup>Low</sup> (blue). Frequencies displayed as a percentage of TCR<sup>Hi</sup> CD5<sup>Hi</sup> CD4<sup>+</sup> SPs.

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## Discussion

FoxP3 plays a central role in establishing and maintaining the  $T_{reg}$  transcriptional program. Therefore deciphering the cues which lead to FoxP3 expression will allow greater understanding of the differentiation and maintenance of the  $T_{reg}$  lineage. In this study we show that a short window of TCR signalling is sufficient for the generation of FoxP3<sup>+</sup>  $T_{reg}$ . Using the tetracycline derivative methacycline, we limited Zap70 expression to the first 48 hours of development. While we saw evidence of death amongst CD4<sup>+</sup> SP cells death in the absence of continued TCR signalling, FoxP3<sup>+</sup>  $T_{reg}$  appeared less vulnerable to death in the absence of Zap70, evident by their progressive enrichment following loss of Zap70. Previous studies reveal the critical role of TCR signalling in the development of  $T_{reg}$ . However the timing and duration of this signal during development still remains unclear. It has been proposed that  $T_{reg}$  development occurs via a TCR dependant phase followed by a cytokine dependant phase of development, however to our knowledge no formal evidence for the temporal separation of these individual signalling events yet exists. In this study we provide evidence detailing the precise temporal requirement for signalling through the TCR during thymic  $T_{reg}$  maturation. We show an early TCR signal is sufficient for FoxP3 induction and that expression is maintained in the absence of a TCR signal. However it remains unclear whether other additional signals are required for FoxP3 expression and development of a stable  $T_{reg}$  lineage.

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In addition to TCR signalling, CD28 co-stimulatory signals have been shown to play a critical role in  $T_{reg}$  differentiation. We confirmed this using CD80/86 blocking antibodies. We demonstrate a 5-fold decrease in thymic  $T_{reg}$  within B1/6 mice treated with a CD80/86 blocking antibody. TetZap70 FoxP3<sup>GFP</sup> chimeras almost completely lacked  $T_{reg}$  altogether when receiving the blocking antibody. Previous work in this area has established a role for CD28-CD80/86 signalling in  $T_{reg}$  differentiation. CD28<sup>-/-</sup> and CD80/86<sup>-/-</sup> mice have marked decreases in  $T_{reg}$  frequencies (Lenschow et al. 2000) (Tai et al. 2005). These are directly comparable to the frequencies observed in B1/6 mice treated with the CD80/86 blocking antibody in our study, confirming the treatment blocking antibody. The studies in CD28<sup>-/-</sup> and CD80/86<sup>-/-</sup> mice focus on adult mice in which the T-cell compartment is in steady state. It therefore remains unclear as to whether the observed difference in the  $T_{reg}$  compartment in these mice are due to homeostatic effects in the periphery or directly affects influencing de novo  $T_{reg}$  development. Our study however demonstrates a direct role for signalling through CD80/86 in thymic FoxP3 induction. CD28 however is not the only signalling molecule reported to interact with the CD80/86 signalling complex, CTLA-4 is also known to interact with CD80/86 and is expressed at high levels on  $T_{reg}$ . It therefore remains possible that the reduction in  $T_{reg}$  frequency observed in the CD80/86 blocked mice was due to blocking CTLA4-CD80/86 interactions. CTLA-4 inhibits T-cell activation through poorly defined mechanisms which may include delivery of inhibitory signals to the T-cell, competition with CD28 for CD80/86 or trans-endocytosis of CD80/86 (Reviewed in Wing, Yamaguchi, & Sakaguchi, 2011)(Qureshi et al. 2011). Although much

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is still unknown about CTLA-4s role in T-cell biology the overriding conclusion thus far is that it plays an inhibitory role limiting T-cell activation. It therefore seems unlikely that the decreased T<sub>reg</sub> frequencies observed in the chimeras which received the CD80/86 blocking antibody were due to blocking CTLA4-CD80/86 interaction, and instead much more likely CD28-CD80/86 interactions play a key role in T<sub>reg</sub> development.

Another form of co-stimulation implicated in T<sub>reg</sub> biology is CD40-CD154 (CD40L) signalling. We found that blocking CD40-CD40L interaction during development had no affect on de novo T<sub>reg</sub> development in TetZap70 FoxP3<sup>GFP</sup> mice. We did however see a reduction in thymic T<sub>reg</sub> in Bl/6 mice after 6 days of CD40-CD40L blockade. Although CD40L-CD40 interactions appear to have no role in de novo T<sub>reg</sub> development, they may instead provide important signals for peripheral T<sub>reg</sub> homeostasis. The T-cell compartment in Bl/6 mice, unlike the TetZap70 FoxP3<sup>GFP</sup> chimeras, is full and under steady state homeostatic control. A contraction of the T<sub>reg</sub> compartment in the periphery could therefore lead to reduced frequencies of T<sub>reg</sub> recirculating back to the thymus. Many previous studies attempt to link reduced thymic T<sub>reg</sub> percentages to defects in de novo T<sub>reg</sub> development, without taking into account recirculant populations. It has been reported that CD40L induces the clonal expansion of FoxP3<sup>+</sup> T<sub>reg</sub> as CD40L blockade led to reduced thymic T<sub>reg</sub> frequencies (Spence & Green 2008). This was later explained by abrogation of the CD40-CD40L signalling pathway inhibiting homeostasis of thymic resident T<sub>reg</sub> (Cuss & Green 2012). Although we cannot rule this out as a possibility, we have shown large

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percentages of T<sub>reg</sub> egress the thymus before re-entering and therefore feel it is much more likely that the reduced thymic T<sub>reg</sub> frequencies observed in these mice are a consequence of reduced recirculation.

After a period of TCR dependant development, our data suggest that T<sub>reg</sub> enter a TCR-independent cytokine phase of maturation. We found no effect on T<sub>reg</sub> frequency in either Bl/6 or TetZap70 FoxP3<sup>GFP</sup> mice treated with a TGFβ blocking antibody. As we observed no phenotype in the Bl/6 mice treated with the TGFβ blocking antibody, there remains the possibility that the TGFβ blockade was not complete in vivo. However the blocking antibody used has been extensively reported to function both in vitro and in vivo at lower doses than those used in this study (Dasch et al. 1989)(X. Zhang et al. 2001). This study therefore casts doubt over a role for TGFβ in de novo T<sub>reg</sub> development, but with the lack of an effective positive control we still cannot be certain if TGFβ signalling has completely ceased. Previous studies into the role of TGFβ in thymic T<sub>reg</sub> development have drawn several conclusions. Using TGFβRII<sup>-/-</sup> OTII TCR transgenic mice it has been shown that TGFβ functions to protect both nT<sub>reg</sub> cells and antigen experienced conventional T-cells from negative selection (Ouyang, Beckett, Ma & M. O. Li 2010). It has also been reported that TGFβ is required to maintain suppressor function in mice expressing a DN TGFβR1 on a CD2 T-cell specific promoter rather than to aid in T<sub>reg</sub> development itself (Marie et al. 2005). However one recent publication in which smad3 binding to the FoxP3 enhancer is removed, showed no requirement for TGFβ signalling in FoxP3 induction in the thymus (Schlenner et al. 2012). These mice contained

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normal numbers of T<sub>reg</sub> and were not at increased susceptibility to colitis. This study would fit with our findings that TGFβ plays no role in T<sub>reg</sub> development maintenance or homeostasis, explaining our inability to observe any T<sub>reg</sub> defects in Bl/6 mice receiving the TGFβ blockade.

IL-2 is strongly implicated in T<sub>reg</sub> development, homeostasis and function. We showed that IL-2 antibody blockade has no effect on de novo development of FoxP3<sup>+</sup> cells in TetZap70 FoxP3<sup>GFP</sup> chimeras. In contrast, blockade in WT mice caused a 50% reduction in the T<sub>reg</sub> numbers. We did however observe a specific requirement for IL-2 signalling in the development of CD4<sup>+</sup> SP CD25<sup>Hi</sup> GFP<sup>+</sup> T<sub>reg</sub>. Development of CD4<sup>+</sup> SP CD25<sup>Low</sup> GFP<sup>+</sup> T<sub>reg</sub> did not require IL-2. Adding an exogenous source of IL-2 to the TetZap70 FoxP3<sup>GFP</sup> chimeras enhanced development of CD4<sup>+</sup> SP CD25<sup>High</sup> GFP<sup>+</sup> T<sub>reg</sub>. The current literature describes a major role for IL-2 in induction of FoxP3 expression and T<sub>reg</sub> development. Hsieh and colleagues propose in their two step model that a strong early TCR signal leads to CD25 up regulation and the formation of a cytokine responsive T<sub>reg</sub> precursor population. CD25<sup>High</sup> FoxP3<sup>-</sup> T<sub>reg</sub> can then respond to paracrine IL-2 in the thymus and begin to express FoxP3 (Chan-Wang Joaquim Lio & C.-S. Hsieh 2008). However in the presence of an IL-2 blockade we see similar percentages of T<sub>reg</sub> to PBS injected control mice. This suggests that the early TCR/co-stimulatory signal is the main driver of FoxP3 expression, while the role of IL-2 maybe is to increase CD25 expression and stabilise the newly developed T<sub>reg</sub>. It remains possible that the IL-2 blockade incompletely abrogated IL-2 signalling or is being compensated for by other γc

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cytokines. However IL-2<sup>-/-</sup> mice are reported to have a 50% reduction in thymic T<sub>reg</sub> (Fontenot, Rasmussen, Gavin & A. Y. Rudensky 2005), a phenotype replicated by the IL-2 blockade treatment in B1/6 mice. A study describing IL-2Rβ<sup>-/-</sup> mice describes the T<sub>reg</sub> compartment comprising predominantly of CD4<sup>+</sup> CD25<sup>-</sup> FoxP3<sup>lo</sup> cells, which expressed low levels of the proliferation marker Ki67 (Cheng et al. 2013). This supports our view that IL-2 is important for maintaining CD25 expression and homeostasis, rather than FoxP3 induction. We therefore suggest TCR and co-stimulatory signals drive initial expression of FoxP3 while IL-2 enhances expression and promotes survival.

The 50% reduction in T<sub>reg</sub> frequency observed in IL-2<sup>-/-</sup> mice could suggest redundancy with other γc cytokines such as IL-15. We found that development of CD4<sup>+</sup> CD25<sup>Lo</sup> FoxP3<sup>+</sup> cells was dependent on IL-15, while development of CD4<sup>+</sup> CD25<sup>Hi</sup> FoxP3<sup>+</sup> cells is dependent on IL-15 & IL-2. Previous studies have shown IL-2Rα<sup>-/-</sup> mice contain relatively normal frequency's of CD4<sup>+</sup> FoxP3<sup>+</sup> T<sub>reg</sub> (Fontenot, Rasmussen, Gavin & A. Y. Rudensky 2005). We also see no effect on T<sub>reg</sub> frequency after IL-2 blockade. In contrast CD122<sup>-/-</sup> (IL-2Rβ) and IL-2<sup>-/-</sup> x IL-15<sup>-/-</sup> mice are reported to exhibit significant decreases in T<sub>reg</sub> frequency (Soper et al. 2007) (M. A. Burchill et al. 2007). This is consistent with the T<sub>reg</sub> frequencies observed in our IL-15R<sup>-/-</sup> chimeras which received the IL-2 blockade. This study coupled with the current literature suggests some redundancy between IL-15 and IL-2 in T<sub>reg</sub> development. However the requirement of the CD4<sup>+</sup> CD25<sup>Lo</sup> FoxP3<sup>+</sup> cells for IL-15 and the CD4<sup>+</sup> CD25<sup>Hi</sup> FoxP3<sup>+</sup> cells for IL-2 has not previously been described. It remains a possibility

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that 2 routes of development of CD4<sup>+</sup> CD25<sup>Hi</sup> FoxP3<sup>+</sup> cells exist, 1 via a CD4<sup>+</sup> CD25<sup>Lo</sup> FoxP3<sup>+</sup> precursor which is dependant on IL-15, and a second via a CD4<sup>+</sup> CD25<sup>Hi</sup> FoxP3<sup>-</sup> precursor which is dependant on IL-2. It also remains possible that this observation is exclusive to development of T<sub>reg</sub> in the TetZap70 FoxP3<sup>GFP</sup> mice, which may lack the correct cytokine milie for efficient T<sub>reg</sub> development. WT mice contain fewer CD4<sup>+</sup> CD25<sup>Lo</sup> FoxP3<sup>+</sup> cells than TetZap70 FoxP3<sup>GFP</sup> mice, perhaps suggesting a more abundant source of thymic IL-2 in WT mice. TetZap70 FoxP3<sup>GFP</sup> mice lack a mature T-cell compartment, and therefore recirculating populations of cells. This could lead to an immature thymic microenvironment and less efficient de novo Treg development.



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## Chapter 5

# The Role of Additional Cell Types in nT<sub>reg</sub> development

### Introduction

FoxP3<sup>+</sup> T<sub>reg</sub> predominantly express the high affinity  $\alpha$ -subunit of the IL-2R, CD25, however the precise role played by IL-2 signalling in T<sub>reg</sub> biology remains contentious. Previous studies find an essential role for IL-2 signalling in the induction of FoxP3 expression during thymic T<sub>reg</sub> development. This is proposed to occur in a 2 step model, strong TCR/co-stimulatory signalling first leads to increased CD25 expression. CD25<sup>HI</sup> FoxP3<sup>-</sup> T-cells are then instructed to express FoxP3 by IL-2 (Chan-Wang Joaquim Lio & C.-S. Hsieh 2008). IL-2<sup>-/-</sup> mice have been shown to contain reduced frequencies of T<sub>reg</sub> and to succumb to lethal auto-immunity (Sadlack et al. 1993). However transfer of highly purified WT CD4<sup>+</sup> CD25<sup>+</sup> T-cells is sufficient to prevent the autoimmunity associated with IL-2 deficiency. This provides direct evidence that the IL-2<sup>-/-</sup> mice have defects in T<sub>reg</sub> production (Malek et al. 2002). However IL-2 independent FoxP3 induction is present in IL-2<sup>-/-</sup> mice which only contain an approximate 50% decrease T<sub>reg</sub> frequencies (Fontenot, Rasmussen, Gavin & A. Y. Rudensky 2005). This casts doubt over a role for IL-2 as the sole signalling cue leading to FoxP3 expression, and suggests a redundancy for IL-2 with other signalling

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events. A previous study has shown IL-2R $\beta$ <sup>-/-</sup> mice contain thymic T<sub>reg</sub> with low expression of CD25, FoxP3 and the proliferation marker Ki67 (Cheng et al. 2013). This suggests a crucial homeostatic role for IL-2 functioning to enhance CD25 and FoxP3 expression as well as stimulating T<sub>reg</sub> proliferation. Genetic mouse models which involve the selective inhibition of IL-2R signalling in the periphery, confirm a role for IL-2 in up-regulating FoxP3 and CD25 in the thymus as well as promoting expansion. These mice contained peripheral T<sub>reg</sub> which were unresponsive to IL-2 and showed slower growth and lower suppressive activity (Bayer et al. 2007). Taken together these studies indicate an important role for IL-2 in T<sub>reg</sub> homeostasis, however it remains uncertain if IL-2 plays a key role in FoxP3 induction during development in the thymus.

IL-2 belongs to the common  $\gamma$ c cytokine family who broadly belong to the class I cytokine receptor superfamily. The IL-2R consists of 3 subunits, IL-2R $\alpha$  (CD25), IL-2R $\beta$  (CD122) and the common gamma chain (CD132). The IL-2R shares both CD122 and  $\gamma$ c subunits with the IL-15R which is additionally comprised of the IL-15R $\alpha$ . The structural similarity between the IL-15R and IL-2R has lead to many studies indicating varying degrees of redundancy existing between the 2 cytokines. The IL-15R comprises of IL-15R $\alpha$ , CD122 and the common gamma chain. CD122<sup>-/-</sup> mice have been shown to contain lower frequencies of thymic T<sub>reg</sub> than IL-2<sup>-/-</sup> mice, implying a contribution by IL-15 signalling in T<sub>reg</sub> biology (M. A. Burchill et al. 2007). However this impairment was not observed in a second study (Fontenot, Rasmussen, Gavin & A. Y. Rudensky 2005). Even though IL-2 and IL-15 contain similar receptor components, IL-15R $\alpha$  deficient

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mice are auto-immune free and contain normal frequencies of FoxP3<sup>+</sup> cells. This suggests IL-2 is the main cytokine involved in T<sub>reg</sub> development and homeostasis.

During steady state conditions IL-2 is produced mainly by CD4<sup>+</sup> SP T<sub>H</sub> cells in secondary lymphoid organs. CD8<sup>+</sup> SPs, NK cells and NKT cells are also capable of IL-2 secretion but to a lesser extent (Reviewed in Boyman & Sprent 2012). IL-2 production is induced in T-cells following activation by antigen (Yang-snyder & Ellen V Rothenberg 1998). However, prolonged exposure to antigen leads to BLIMP1 expression which progressively reduces the capacity of these cells to secrete IL-2 (Kallies et al. 2009). IL-2 reporter mice show IL-2 secretion isolated to 3 major sites, the thymus, the skin and the gut. Within these mice TCRαβ and TCRγδ T-cells are described as secreting 'halos' of IL-2 which diffuse over many cell diameters (Yang-snyder & Ellen V Rothenberg 1998). The pattern of expression for IL-2 seems to indicate expression is confined to contexts where T-cells are stimulated by antigen. Expression within the thymus suggests the possibility of IL-2 secretion occurring during negative selection, upon exposure to self-antigens. This is supported by observations of IL-2 being detected during T-cell apoptosis (Bassiri & Carding 2001). Therefore negative selection within the thymus and the establishment of central tolerance may play an additional role in maintaining peripheral tolerance also.

The thymic stroma has been shown to be crucial for both central and peripheral tolerance. cTECs and mTECs differentiate from bipotent progenitors present in the embryonic and postnatal thymus (Rossi et al. 2006). Currently experimental

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evidence favors the terminal differentiation model for mTEC development (Kyewski & Klein 2006)(Gillard & Farr 2005). According to this model mTECs are thought to be in various stages of development, constantly proliferating, with less mature mTECs gradually replacing a short lived fully mature population. The most mature mTECs are thought to be characterised by high levels of Aire, CD80/86, CD40 and MHCII (Gray et al. 2007). The gradual maturation of the mTECs is also thought to be associated with an expansion of TSA repertoire. Maturation of mTECs is proposed to occur via the nuclear factor kappa B (NfκB) in both the canonical and non-canonical pathways. This occurs via activation of CD40 and RANK by CD40L and RANKL present on mature SP thymocytes (Akiyama et al. 2008), as well as lymphotoxin β signals from positively selected T-cells (White et al. 2010). The presence of mature SP thymocytes are therefore heavily implicated in the maturation of the thymic medulla and therefore both central and peripheral tolerance. We therefore examined the effect on de novo T<sub>reg</sub> development in TetZap70 FoxP3<sup>GFP</sup> chimeras which already contained a fully reconstituted lymphoid compartment. This allowed us to examine T<sub>reg</sub> development in mice which contain a fully reconstituted T-cell compartment and therefore a fully mature thymic stroma. We found no effect on the timing or frequency of T<sub>reg</sub> which develop in these mice. But instead found that a hematopoietic source of IL-2 is required for CD25<sup>Hi</sup> FoxP3<sup>+</sup> T<sub>reg</sub> development.

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## Results

### Increase CD25 expression in TetZap70 FoxP3<sup>GFP</sup> mixed chimeras

Un-induced TetZap70 FoxP3<sup>GFP</sup> mice are lymphopenic. The presence of a mature CD4<sup>+</sup> SP compartment has been shown to be critical for full maturation of the thymic stroma (White et al. 2010)(Gill et al. 2008; Hikosaka et al. 2008). A fully mature stroma is heavily implicated in FoxP3 expression and the induction of central tolerance (Cowan et al. 2013). Therefore the lack of a mature CD4<sup>+</sup> SP compartment in TetZap70 FoxP3<sup>GFP</sup> mice may suggest an immature thymic microenvironment leading to inefficient T<sub>reg</sub> development. In order to investigate this we made mixed bone marrow chimeras in which irradiated Rag1<sup>-/-</sup> hosts were injected with TetZap70 FoxP3<sup>GFP</sup> and T-cell depleted B1/6 CD45.1 bone marrow in a 1:1 ratio (B1/6 : TetZap70 FoxP3<sup>GFP</sup> mixed chimeras here on in). These mice were then left for 6 weeks to reconstitute before being fed dox and their thymus analysed by FACs. Zap70 induction in these mice would therefore be in the context of pre-existing thymic development from the WT partner cells. GFP<sup>+</sup> cells were first detected on day 4 in both mixed chimeras and single chimera controls (Fig 3.1 A&B). The total GFP<sup>+</sup> cell frequency as a percentage of CD4<sup>+</sup> SPs and total thymus increased steadily throughout the time course until day 8, (Fig 3.1B). Total CD4<sup>+</sup> SP GFP<sup>+</sup> cell percentages were similar in both single and mixed chimeras. As described previously TetZap70 FoxP3<sup>GFP</sup> single chimeras specifically lacked a CD4<sup>+</sup> SP GFP<sup>+</sup> CD25<sup>High</sup> compartment until day 5, at which point it constituted a very small percentage of total GFP<sup>+</sup> cells. However beginning with their detection on day 4, the GFP<sup>+</sup> compartment

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within the mixed chimeras was dominated by CD4<sup>+</sup> SP GFP<sup>+</sup> CD25<sup>High</sup> cells. The ratio of CD25<sup>High</sup>:CD25<sup>Lo</sup> cells within the GFP<sup>+</sup> compartment of these mice was higher than single chimeras throughout development (Fig 3.2). Therefore the presence of WT Bl/6 development in Bl/6 : TetZap70 FoxP3<sup>GFP</sup> mixed chimeras permitted more efficient development of CD4<sup>+</sup> SP GFP<sup>+</sup> CD25<sup>High</sup> T<sub>reg</sub> cells without appearing to affect the timing or overall magnitude of T<sub>reg</sub> development.

### **T<sub>reg</sub> exit the thymus on day 7 in TetZap70 FoxP3<sup>GFP</sup> mixed chimeras**

We next investigated the timing of T<sub>reg</sub> emigration to the periphery in Bl/6 : TetZap70 FoxP3<sup>GFP</sup> mixed chimeras. In order to achieve this Bl/6 : TetZap70 FoxP3<sup>GFP</sup> mixed chimeras were fed dox for 1-13 days and their thymus and LNs analysed by FACs. GFP<sup>+</sup> T<sub>reg</sub> were first detected in the LNs approximately 7 days after dox feeding (Fig 3.3B). Interestingly CD4<sup>+</sup> SP GFP<sup>+</sup> CD25<sup>Lo</sup> cells were equally able to migrate from the thymus to the LNs as CD4<sup>+</sup> SP GFP<sup>+</sup> CD25<sup>Hi</sup> cells in both TetZap70 FoxP3<sup>GFP</sup> mixed chimeras and single chimera controls. The ratio of CD25<sup>Hi</sup> : CD25<sup>Lo</sup> cells present in the thymus during development was maintained in the periphery in mixed and single chimeras (Fig 3.3A). We also observed increased GFP<sup>+</sup> T<sub>reg</sub> percentages in the periphery of Bl/6 : TetZap70 FoxP3<sup>GFP</sup> mixed chimeras (Fig 3.3A). Therefore the presence of WT Bl/6 cells in mixed chimeras has no effect on the timing of T<sub>reg</sub> emigration to the periphery. CD25 expression levels on de novo generated T<sub>reg</sub> also appear to have no effect on their subsequent ability to emigrate to the periphery, where

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we see increased GFP<sup>+</sup> T<sub>reg</sub> percentages in mixed chimeras compared to single chimera controls.

### **Mixed chimeras contain thymic recirculants and newly developed T-cells**

Since B1/6 bone marrow in mixed chimeras allows induction of larger percentages of CD4<sup>+</sup> SP GFP<sup>+</sup> CD25<sup>Hi</sup> T<sub>reg</sub>, we next aimed to investigate the thymic composition of the mixed chimeras. We investigated the expression of HSA, Qa-2 and CD62L maturation markers in an attempt to determine the proportion of the various T-cell compartments contained within the mixed chimeras which had re-circulated from the periphery. The vast majority of CD45.1<sup>+</sup> B1/6 CD4<sup>+</sup> SPs within the mixed chimeras showed high Qa-2 and low HSA expression levels (Fig 3.4A). This indicates the presence of a mature T-cell compartment which has most likely re-circulated back to the thymus after maturation in the periphery. The CD45.1<sup>-</sup> TetZap70 FoxP3<sup>GFP</sup> cells however exhibited primarily low surface expression of Qa-2 while maintaining high HSA levels (Fig 3.4A). This is consistent with newly developed T-cells which are yet to fully mature and exit the thymus. However newly developed CD45.1<sup>-</sup> TetZap70 FoxP3<sup>GFP</sup> cells have already begun to express Qa-2 and down regulate HSA expression. Therefore these 2 markers alone are insufficient to exclusively distinguish between newly developed and recirculating populations of T-cells. Expression of CD62L however appears to be confined primarily to newly developed T<sub>reg</sub>. CD45.1<sup>+</sup> B1/6 CD4<sup>+</sup> SPs T<sub>reg</sub> exhibit bimodal expression of CD62L while de novo developed CD45.1<sup>-</sup> TetZap70 FoxP3<sup>GFP</sup> cells express high levels of CD62L (Fig 3.4B). Taken together this indicates B1/6 : TetZap70

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FoxP3<sup>GFP</sup> mixed bone marrow chimeras contain mature thymic T-cell populations which are not present in TetZap70 FoxP3<sup>GFP</sup> single chimeras. These T-cells are HSA<sup>Low</sup> and Qa-2<sup>High</sup> but also appear to have low expression of CD62L.

### **IL-2 blockade in mixed chimeras prevent GFP<sup>+</sup> CD25<sup>High</sup> T<sub>reg</sub> development**

Next we wished to investigate the signalling cues responsible for the development of the prominent CD4<sup>+</sup> GFP<sup>+</sup> CD25<sup>Hi</sup> T<sub>reg</sub> in the Bl/6 : TetZap70 FoxP3<sup>GFP</sup> mixed chimeras. As IL-2 is shown to both initiate FoxP3 expression and increase CD25 expression levels, we investigated if a thymic source of IL-2 was responsible for the increased development of CD4<sup>+</sup> GFP<sup>+</sup> CD25<sup>Hi</sup> T<sub>reg</sub> in mixed chimeras. In order to investigate this Bl/6 : TetZap70 FoxP3<sup>GFP</sup> mixed chimeras along with Bl/6 controls were treated with an IL-2 blocking antibody every 2 days commencing 1 day before being fed dox. Their thymus was then analysed on day 6 by FACs. Bl/6 mice which received the IL-2 blockade exhibited an approximate 50% decrease in CD4<sup>+</sup> GFP<sup>+</sup> CD25<sup>Hi</sup> T<sub>reg</sub> while CD4<sup>+</sup> GFP<sup>+</sup> CD25<sup>Lo</sup> frequency's were maintained when compared to PBS injected controls (Fig 3.5A). Bl/6 : TetZap70 FoxP3<sup>GFP</sup> mixed chimeras which received the IL-2 blockade completely lacked CD4<sup>+</sup> SP GFP<sup>+</sup> CD25<sup>Hi</sup> T<sub>reg</sub>. In contrast PBS injected controls maintained development of a prominent CD4<sup>+</sup> GFP<sup>+</sup> CD25<sup>Hi</sup> compartment (Fig 3.5 B&C). Therefore development of CD4<sup>+</sup> GFP<sup>+</sup> CD25<sup>Hi</sup> T<sub>reg</sub> in TetZap70 FoxP3<sup>GFP</sup> chimeras requires IL-2 and argues that the thymic source of IL-2 is more abundant in chimeras containing WT cells compared to TetZap70 FoxP3<sup>GFP</sup> single chimeras.



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## **CD40-CD40L signalling does not affect T<sub>reg</sub> frequencies in mixed chimeras**

It has been shown that abrogation of CD40-CD40L signalling impedes the homeostasis of thymic T<sub>reg</sub> through altering IL-2 levels (Cuss & Green 2012). It was therefore possible that signalling through CD40 by WT Bl/6 cells was responsible for increased thymic IL-2 present in Bl/6 : TetZap70 FoxP3<sup>GFP</sup> mixed chimeras. We therefore decided to investigate a role for CD40-CD40L signalling in de novo T<sub>reg</sub> development in Bl/6 : TetZap70 FoxP3<sup>GFP</sup> mixed chimeras. To do this, we treated mixed chimeras with 0.5mg of a CD40L blocking antibody (MR1). The Bl/6 : TetZap70 FoxP3<sup>GFP</sup> chimeras received an injection of the blocking antibody every 2 days commencing one day before being fed dox. Their thymus was then analysed on day 6 by FACs. We observed a 2-fold decrease in thymic Bl/6 CD45.1<sup>+</sup> CD4<sup>+</sup> SP CD44<sup>Int</sup> CD25<sup>Hi</sup> T<sub>reg</sub> within mice which received the CD40-CD40L blockade (Fig 3.6). We also observed a significant but more modest decrease in Bl/6 CD45.1<sup>+</sup> CD4<sup>+</sup> SP CD44<sup>Int</sup> CD25<sup>High</sup> T<sub>reg</sub> percentages within the LNs However we saw no effect on CD45.1<sup>-</sup> CD4<sup>+</sup> SP GFP<sup>+</sup> de novo T<sub>reg</sub> development (Fig 3.6A).. Frequencies of total GFP<sup>+</sup> cells remained unchanged with or without CD40L blockade, as well as the distribution of CD25 expression levels within the GFP<sup>+</sup> compartment (Fig 3.6 A&B). CD40L blockade did therefore not alter the development of CD45.1<sup>+</sup> CD4<sup>+</sup> SP CD25<sup>High</sup> T<sub>reg</sub> in mixed chimeras. We therefore provide further evidence for a homeostatic role for CD40-CD40L signalling in the periphery rather than a role in de novo T<sub>reg</sub> development. We also see no evidence for CD40L-CD40 signalling in regulating thymic IL-2 through observations of

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unchanged CD25 expression levels on developing T<sub>reg</sub>, a process which we have shown to be IL-2 dependant (Fig 3.5).

**T<sub>reg</sub> develop in the absence of IL-2 and IL-15 signalling in mixed chimeras.**

We previously demonstrated a requirement for IL-2 and IL-15 during de novo T<sub>reg</sub> development in TetZap70 FoxP3<sup>GFP</sup> single chimeras. We have shown a stromal source of IL-15 is important for T<sub>reg</sub> development in these mice. It was therefore possible that stromal maturation by Bl/6 T-cells in Bl/6 : TetZap70 FoxP3<sup>GFP</sup> mixed chimeras was enhancing stromal IL-15 signalling. Due to a requirement for the IL-15R $\alpha$  to be trans-presented by the thymic stroma for IL-15 signalling during T-cell development, we took advantage of IL-15R $\alpha$ <sup>-/-</sup> mice which cannot provide IL-15 in vivo. Rag1<sup>-/-</sup> IL-15R $\alpha$ <sup>-/-</sup> mice were sub-lethally irradiated and injected with T-cell depleted Bl/6 CD45.1<sup>+</sup> and TetZap70 FoxP3<sup>GFP</sup> bone marrow in a 1:1 ratio, (Bl/6 : TetZap70 FoxP3<sup>GFP</sup> - Rag1<sup>-/-</sup> IL-15R $\alpha$ <sup>-/-</sup> host mixed chimeras here on in). Chimeras were left for 6 weeks to reconstitute before being fed dox and analysed by FACs 6 days later. Groups of Bl/6 : TetZap70 FoxP3<sup>GFP</sup> - Rag1<sup>-/-</sup> IL-15R $\alpha$ <sup>-/-</sup> host mixed chimeras along with Bl/6 : TetZap70 FoxP3<sup>GFP</sup> mixed chimeras controls were then treated with an IL-2 blocking antibody every 2 days, commencing one day before being fed dox. Thymic T<sub>reg</sub> frequencies in Bl/6 : TetZap70 FoxP3<sup>GFP</sup> - Rag1<sup>-/-</sup> IL-15R $\alpha$ <sup>-/-</sup> host mixed chimeras were unchanged from Bl/6 : TetZap70 FoxP3<sup>GFP</sup> mixed chimeras controls (Fig 3.7 A&B). Upon treatment with an IL-2 blocking antibody the prominent, GFP<sup>+</sup> CD25<sup>High</sup> T<sub>reg</sub> population normally present in mixed chimeras was completely removed, in both sets of chimeras (Fig 3.7 A&C). The

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abrogation of IL-2 signalling completely blocked development of CD4<sup>+</sup> SP GFP<sup>+</sup> CD25<sup>Hi</sup> T<sub>reg</sub> (Fig 3.7 A&C). We therefore show no effect on T<sub>reg</sub> development in Bl/6 : TetZap70 FoxP3<sup>GFP</sup> - Rag1<sup>-/-</sup> IL-15Rα<sup>-/-</sup> host mixed chimeras. Suggesting no role for T-cell dependant maturation of the thymic stroma contributing to development via IL-15 signalling. Instead we further confirm an essential role for IL-2 signalling in CD4<sup>+</sup> SP CD25<sup>Hi</sup> T<sub>reg</sub> development.

### **No evidence that stroma provide IL-2 for T<sub>reg</sub> development**

We have shown a role for the thymic stroma in IL-15 signalling during de novo T<sub>reg</sub> development in single chimeras (Fig 2.8). We wondered if the thymic stroma could also be a source of IL-2. To investigate this we sub-lethally irradiated Rag1<sup>-/-</sup> IL-2<sup>-/-</sup> mice and injected them with TetZap70 FoxP3<sup>GFP</sup> bone marrow (TetZap70 FoxP3<sup>GFP</sup> IL-2<sup>-/-</sup> host chimeras here on in). These mice were then left for 6 weeks to reconstitute before being fed dox and their thymus analysed by FACs after 6 days. The TetZap70 FoxP3<sup>GFP</sup> IL-2<sup>-/-</sup> host chimeras contained normal percentages of CD4<sup>+</sup> SP and CD8<sup>+</sup> SP T-cells (Fig 3.8A). Frequencies of CD4<sup>+</sup> SP GFP<sup>+</sup> T<sub>reg</sub> were also similar to TetZap70 FoxP3<sup>GFP</sup> Rag1<sup>-/-</sup> host chimera controls, CD25 expression levels on CD4<sup>+</sup> SP GFP<sup>+</sup> T<sub>reg</sub> were also unaltered (Fig 3.8B). We therefore found no evidence for a stromal source of IL-2. Since the stroma appeared not to be a crucial source of IL-2 during de novo T<sub>reg</sub> development, we investigated whether there was a haematopoietic source of IL-2. In order to achieve this we injected Rag1<sup>-/-</sup> hosts with TetZap70 FoxP3<sup>GFP</sup> and IL-2<sup>-/-</sup> bone marrow in a 1:1 ratio (CD45.1<sup>+</sup> IL-2<sup>-/-</sup> : TetZap70 FoP3<sup>GFP</sup> mixed chimeras here on in). After 6 weeks the CD45.1<sup>+</sup> IL-2<sup>-/-</sup>

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<sup>-/-</sup> : TetZap70 FoP3<sup>GFP</sup> mixed chimeras were fed dox and their thymus analysed by FACs. T<sub>reg</sub> frequencies within the IL-2<sup>-/-</sup> CD45.1<sup>+</sup> compartment in CD45.1<sup>+</sup> IL-2<sup>-/-</sup> : TetZap70 FoP3<sup>GFP</sup> mixed chimeras were reduced compared to Bl/6 CD45.1<sup>+</sup> : TetZap70 FoP3<sup>GFP</sup> mixed chimera controls (Fig 3.9). This is consistent with previous observations made regarding T<sub>reg</sub> frequencies in IL-2<sup>-/-</sup> mice (Fontenot, Rasmussen, Gavin & A. Y. Rudensky 2005). CD45.1<sup>+</sup> IL-2<sup>-/-</sup> : TetZap70 FoP3<sup>GFP</sup> mixed chimeras contained reduced frequencies of CD45.1<sup>+</sup> GFP<sup>+</sup> T<sub>reg</sub>. The GFP<sup>+</sup> de novo T<sub>reg</sub> within the chimeras also expressed much lower levels of CD25 when compared to Bl/6 control chimeras (Fig 3.9A). This suggests a hematopoietic source of IL-2 for de novo T<sub>reg</sub> development. However we noted that CD45.1<sup>+</sup> IL-2<sup>-/-</sup> : TetZap70 FoP3<sup>GFP</sup> mixed chimeras also contained an enlarged CD4<sup>+</sup> SP T<sub>mem</sub> cell compartment along with signs of thymic involution. This may be a result of autoimmunity due to peripheral reconstitution with IL-2<sup>-/-</sup> cells. The effects of this on de novo T<sub>reg</sub> development can therefore not be excluded. Taken together these data further suggest a hematopoietic source of IL-2 which is necessary for efficient CD4<sup>+</sup> SP GFP<sup>+</sup> CD25<sup>Hi</sup> T<sub>reg</sub> development.

### **TCR transgenic T-cells do not enhance GFP<sup>+</sup> CD25<sup>High</sup> T<sub>reg</sub> development**

Next we aimed to identify the cell type within the hematopoietic compartment which was responsible for enhancing development of CD4<sup>+</sup> SP GFP<sup>+</sup> CD25<sup>Hi</sup> T<sub>reg</sub> in the TetZap70 FoxP3<sup>GFP</sup> mixed chimeras. IL-2 is reported to be produced in activated CD4<sup>+</sup> SP and CD8<sup>+</sup> SP and to a lesser extent activated DCs (Reviewed in Malek 2008). We therefore took advantage of OT1 and OTII TCR

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transgenic mice which are MHCII and MHCII restricted expressing TCR specific for OVA peptide.

Developing T-cells within these mice are restricted to the CD8<sup>+</sup> SP and CD4<sup>+</sup> SP lineage respectively. In order to investigate the role played by CD4<sup>+</sup> SP T-cells in T<sub>reg</sub> development we made mixed bone marrow chimeras containing TetZap70 FoxP3<sup>GFP</sup> and either OTII Rag1<sup>-/-</sup> or OTI Rag1<sup>-/-</sup> bone marrow in a 1:1 ratio (OTI/OTII : TetZap70 FoxP3<sup>GFP</sup> mixed chimeras here on in). OTII : TetZap70 FoxP3<sup>GFP</sup> mixed chimeras and TetZap70 FoxP3<sup>GFP</sup> single chimera controls were left for 6 weeks to reconstitute before being fed dox and their thymus analysed by FACS. OTII : TetZap70 FoxP3<sup>GFP</sup> mixed chimeras contained approximately 50% Vα2<sup>+</sup> HuCD2<sup>-</sup> OTII T-cells, which were restricted to the CD4<sup>+</sup> SP lineage (Fig 3.10A). When excluding Vα2<sup>+</sup> HuCD2<sup>-</sup> CD4<sup>+</sup> SP OTII T-cells, we observed no effect on CD4<sup>+</sup> SP GFP<sup>+</sup> T<sub>reg</sub> development in OTII mixed bone marrow chimeras compared to TetZap70 FoxP3<sup>GFP</sup> single chimera controls. CD25 expression levels on GFP<sup>+</sup> cells remained lower than in Bl/6 : TetZap70 FoxP3<sup>GFP</sup> mixed chimeras in both sets of mice (Fig 3.10B). Generating similar chimeras using OTI bone marrow generated similar observations (Fig 3.10C). These data suggest that solely restoring positive selection of either CD4<sup>+</sup> SP or CD8<sup>+</sup> SP T-cells is not sufficient to support CD4<sup>+</sup> SP GFP<sup>+</sup> CD25<sup>Hi</sup> T<sub>reg</sub> development by IL-2 secretion. As OTI and OTII TCR transgenic T-cells fail to undergo negative selection, this would suggest a possible role for negative selection in the supply sufficient quantities of thymic IL-2. However this data is also consistent with the requirement for an additional

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hematopoietic cell type which is not present in the OTII : TetZap70 FoxP3<sup>GFP</sup> mixed chimeras such as T<sub>reg</sub> of T<sub>mem</sub> cells.

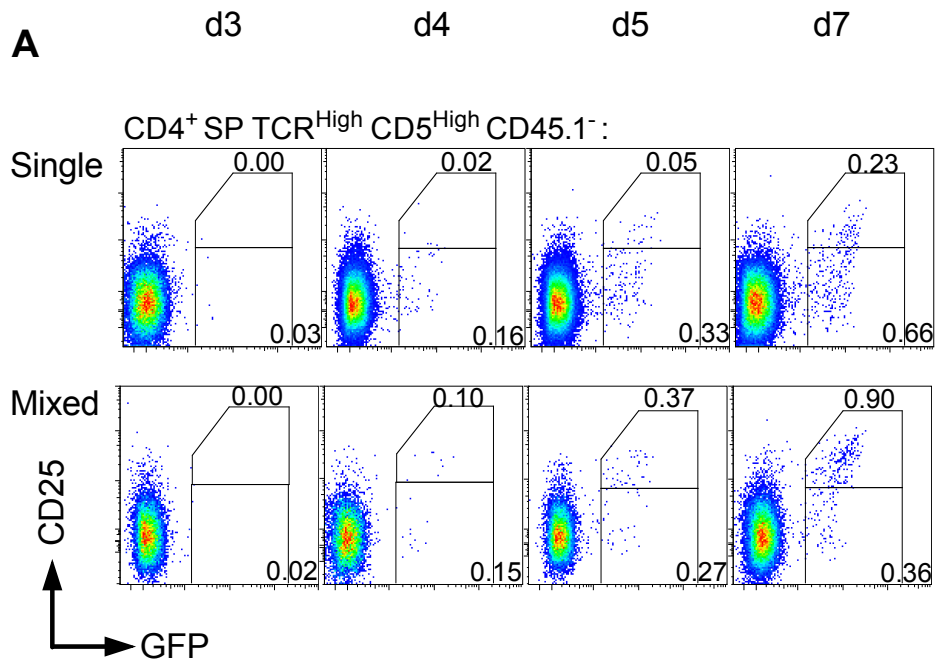
### **IL-2 dependant CD25<sup>Hi</sup> T<sub>reg</sub> development does not require T<sub>mem</sub> or T<sub>reg</sub>**

We have shown that significant proportions of thymic T-cells have re-circulated from the periphery. As T-cell activation accompanies IL-2 production (Naramura et al. 1998) we next investigated the recirculation of activated T-cell populations as a source of IL-2 for de novo T<sub>reg</sub> development. To achieve this we took advantage of IKK2<sup>FI/FI</sup> CD4 Cre R26 mice. These contain naïve CD4<sup>+</sup> SP T-cells but lack both T<sub>mem</sub> and T<sub>reg</sub> cells. We injected Rag1<sup>-/-</sup> mice with TetZap70 FoxP3<sup>GFP</sup> and IKK2<sup>FI/FI</sup> CD4 Cre R26 or IKK2<sup>FI/WT</sup> R26 bone marrow in a 1:1 ratio (IKK2<sup>FI/FI</sup> : TetZap70 FoxP3<sup>GFP</sup> mixed chimeras here on in). IKK2<sup>FI/FI</sup> : TetZap70 FoxP3<sup>GFP</sup> mixed chimeras therefore allow analysis of de novo T<sub>reg</sub> development in the presence of polyclonal CD4<sup>+</sup> and CD8<sup>+</sup> SPs while excluding T<sub>mem</sub> and T<sub>reg</sub> cells. After 6 weeks reconstitution chimeras were fed dox and their thymus analysed by FACs. As expected YFP<sup>+</sup> CD4<sup>+</sup> SP cells completely lacked CD44<sup>Int</sup> CD25<sup>Hi</sup> T<sub>reg</sub> along with CD44<sup>Hi</sup> CD25<sup>Lo</sup> T<sub>mem</sub> cells when compared to IKK<sup>FI/WT</sup> mixed chimera controls (Fig 3.11). YFP<sup>-</sup> cells in both IKK2<sup>FI/FI</sup> and IKK2<sup>FI/WT</sup> TetZap70 FoxP3<sup>GFP</sup> mixed chimeras contained large percentages of CD4<sup>+</sup> SP GFP<sup>+</sup> CD25<sup>Hi</sup> T<sub>reg</sub> (Fig 3.11). These data indicate that T<sub>mem</sub> and T<sub>reg</sub> cells are not required for the IL-2 dependant generation of CD4<sup>+</sup> SP GFP<sup>+</sup> CD25<sup>Hi</sup> T<sub>reg</sub>. This is also consistent but not indicative of a requirement for a polyclonal T-cell compartment for efficient T<sub>reg</sub> induction. T-cells in IKK2<sup>FI/FI</sup> : TetZap70 FoxP3<sup>GFP</sup> mixed chimeras contain polyclonal TCRs. Therefore unlike

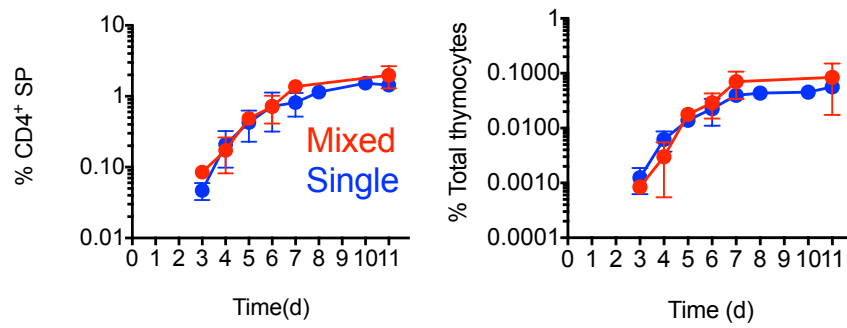
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the TCR transgenic OTI/OTII : TetZap70 FoxP3<sup>GFP</sup> mixed chimeras cells in the thymus of IKK2 Fl/Fl : TetZap70 FoxP3<sup>GFP</sup> mixed chimeras are undergoing negative selection. As IL-2 secretion occurs upon activation of the TCR, IL-2 production in the thymus may reflect T-cells undergoing negative selection as T-cell activation accompanies this (Naramura et al. 1998).

### 3.1



### B



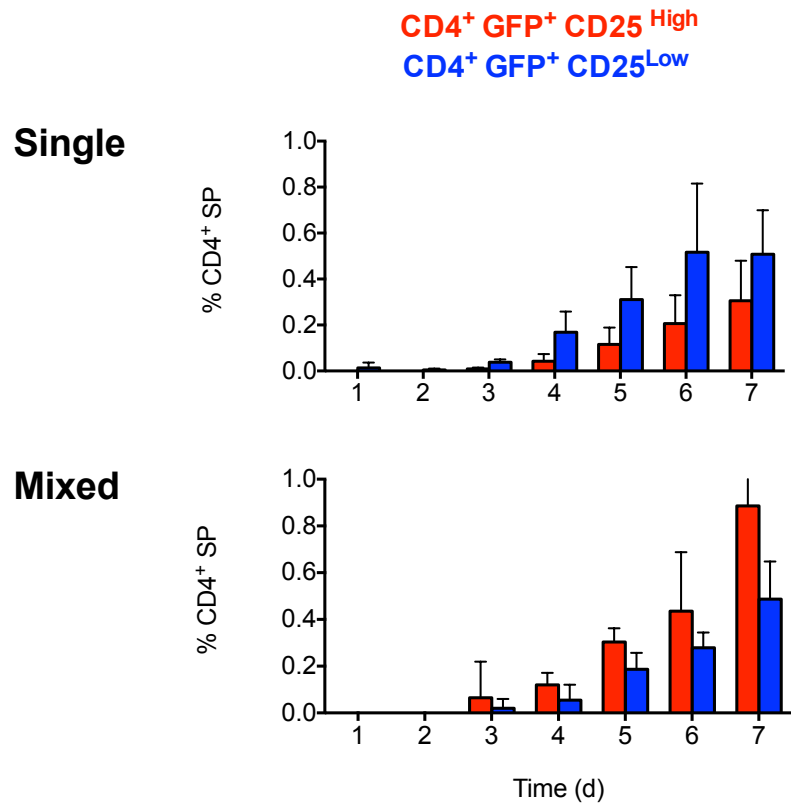


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**Figure 3.1 Bl/6 CD45.1:TetZap70 FoxP3<sup>GFP</sup> mixed chimeras.**

Rag1<sup>-/-</sup> mice were irradiated and injected with 3M T-cell depleted Bl/6 CD45.1 bone marrow cells along with 3M TetZap70 FoxP3<sup>GFP</sup> bone marrow cells in a single iv injection. Alternatively Rag1<sup>-/-</sup> mice were injected with 3M TetZap70 FoxP3<sup>GFP</sup> bone marrow cells alone, as controls. Bone marrow was harvested, T-cell depleted and injected as described in materials and methods. The resulting chimeras were left for 6 weeks to reconstitute before being fed dox for 3-7 days and analysed by FACs. **A:** FACs plots comparing CD25 and GFP expression on CD45.1<sup>-</sup> TCR<sup>Hi</sup> CD5<sup>Hi</sup> CD4<sup>+</sup> SP thymocytes from TetZap70 FoxP3<sup>GFP</sup> single chimeras (Top panel) or Bl/6 CD45.1 : TetZap70 FoxP3<sup>GFP</sup> mixed chimeras (bottom panel), after 3-7 days of dox feeding. **B:** Line graphs comparing CD45.1<sup>-</sup> TCR<sup>Hi</sup> CD5<sup>Hi</sup> CD4<sup>+</sup> SP GFP<sup>+</sup> frequency in TetZap70 FoxP3<sup>GFP</sup> single chimeras (blue) or Bl/6 CD45.1 : TetZap70 FoxP3<sup>GFP</sup> mixed chimeras (red). Frequencies expressed as a percentage of TCR<sup>Hi</sup> CD5<sup>Hi</sup> CD4<sup>+</sup> SPs (left) or as a percentage of total thymocytes (right).

### 3.2



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**Figure 3.2 Prominent GFP<sup>+</sup> CD25<sup>Hi</sup> compartment in mixed chimeras.**

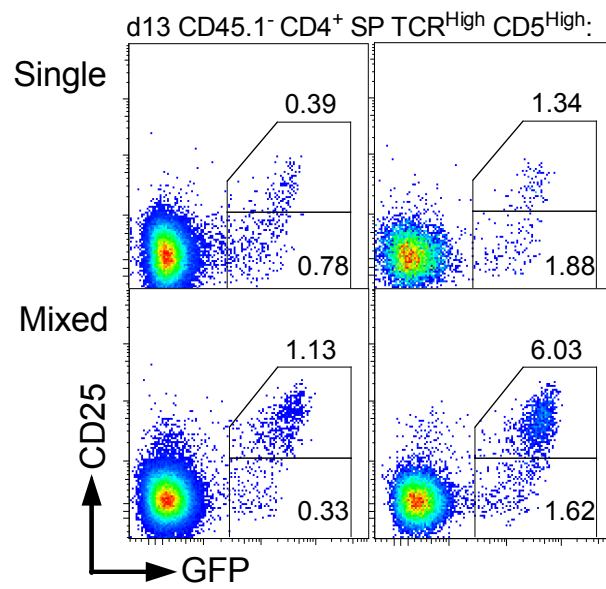
Rag1<sup>-/-</sup> mice were irradiated and injected with 3M T-cell depleted Bl/6 CD45.1 bone marrow cells along with 3M TetZap70 FoxP3<sup>GFP</sup> bone marrow cells in a single iv injection. Alternatively Rag1<sup>-/-</sup> mice were injected with 3M TetZap70 FoxP3<sup>GFP</sup> bone marrow cells alone, as controls. Bone marrow was harvested, T-cell depleted and injected as described in materials and methods. The resulting chimeras were left for 6 weeks to reconstitute before being fed dox for 0-7 days and analysed by FACs. Bar graphs showing the frequency of TCR<sup>Hi</sup> CD5<sup>Hi</sup> CD4<sup>+</sup> SP GFP<sup>+</sup> CD25<sup>Hi</sup> cells (red) or TCR<sup>Hi</sup> CD5<sup>Hi</sup> CD4<sup>+</sup> SP GFP<sup>+</sup> CD25<sup>Lo</sup> cells (blue) as a percentage of TCR<sup>Hi</sup> CD5<sup>Hi</sup> CD4<sup>+</sup> SPs each day after dox feeding, for the TetZap70 FoxP3<sup>GFP</sup> single chimeras (top) and Bl/6 CD45.1: TetZap70 FoxP3<sup>GFP</sup> mixed chimeras (bottom).

### 3.3

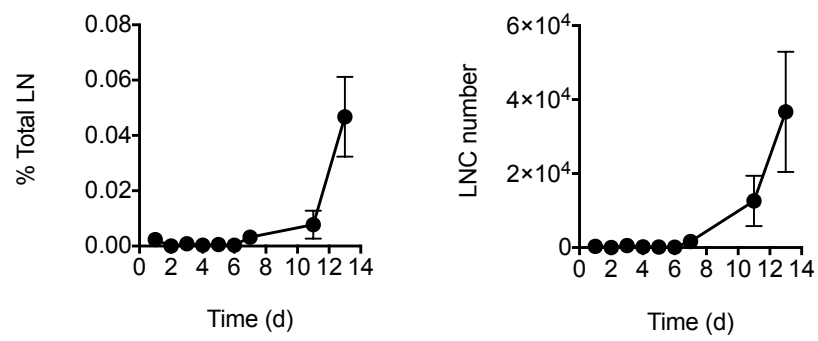
Thymus

LN

**A**



**B**

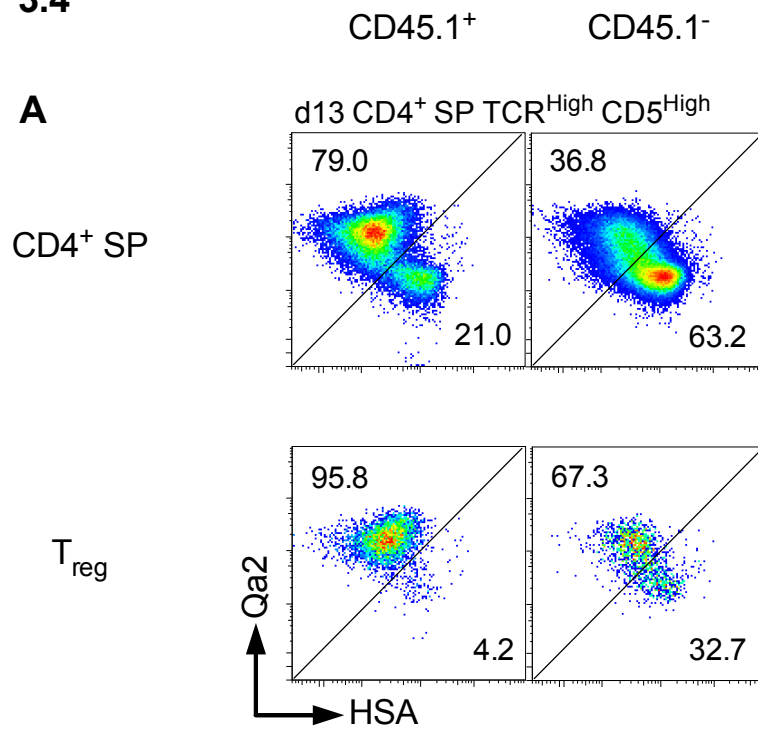


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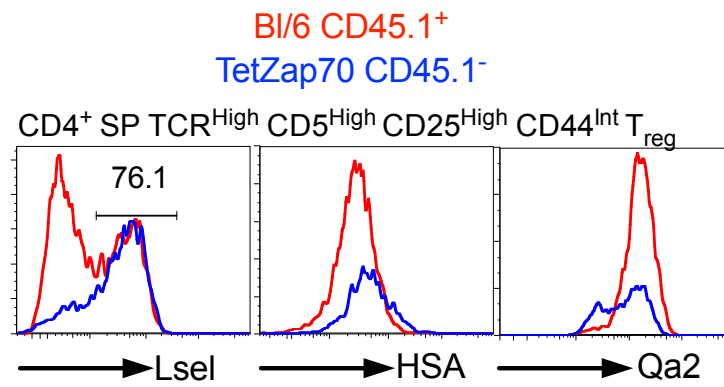
**Figure 3.3 GFP<sup>+</sup> CD25<sup>Hi</sup> and CD25<sup>Lo</sup> cells in mixed chimera LNs.**

Rag1<sup>-/-</sup> mice were irradiated and injected with 3M T-cell depleted B1/6 CD45.1 bone marrow cells along with 3M TetZap70 FoxP3<sup>GFP</sup> bone marrow cells in a single iv injection. Alternatively Rag1<sup>-/-</sup> mice were injected with 3M TetZap70 FoxP3<sup>GFP</sup> bone marrow cells alone, as controls. Bone marrow was harvested, T-cell depleted and injected as described in materials and methods. The resulting chimeras were left for 6 weeks to reconstitute before being fed dox for 0-13 days before analysis of thymocytes and LN cells by FACs. **A:** FACs plots comparing CD25 and GFP expression on thymocytes (left) and LN cells (right) of CD45.1<sup>-</sup> TCR<sup>Hi</sup> CD5<sup>Hi</sup> CD4<sup>+</sup> SP cells from TetZap70 FoxP3<sup>GFP</sup> single chimeras (Top panel) or B1/6 CD45.1 : TetZap70 FoxP3<sup>GFP</sup> mixed chimeras (bottom panel). **B:** Line graphs comparing CD45.1<sup>-</sup> TCR<sup>Hi</sup> CD5<sup>Hi</sup> CD4<sup>+</sup> SP GFP<sup>+</sup> LN cell frequency B1/6 CD45.1 : TetZap70 FoxP3<sup>GFP</sup> mixed chimeras. Frequencies expressed as a percentage of total TCR<sup>Hi</sup> CD5<sup>Hi</sup> CD4<sup>+</sup> SPs LN cells (left) and absolute Ln cell number (right).

3.4



**B**



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### Figure 3.4 Mixed chimeras contain mature recirculant T-cell populations

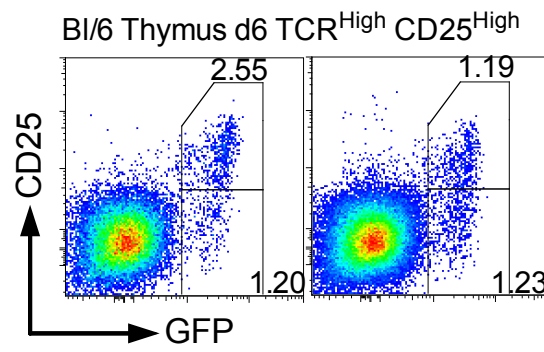
Rag1<sup>-/-</sup> mice were irradiated and injected with 3M T-cell depleted Bl/6 CD45.1 bone marrow cells along with 3M TetZap70 FoxP3<sup>GFP</sup> bone marrow cells in a single iv injection. Bone marrow was harvested, T-cell depleted and injected as described in materials and methods. The resulting chimeras were left for 6 weeks to reconstitute and fed dox for 13 days before the thymus was analysed by FACs. **A:** FACs plots comparing HSA and Qa-2 expression on TCR<sup>Hi</sup> CD5<sup>Hi</sup> CD4<sup>+</sup> SPs (top) and TCR<sup>Hi</sup> CD5<sup>Hi</sup> CD4<sup>+</sup> SP GFP<sup>+</sup> T<sub>reg</sub> (bottom) from Bl/6 CD45.1<sup>+</sup> cells (left) or CD45.1<sup>-</sup> TetZap70 FoxP3<sup>GFP</sup> cells (right) from within the same Bl/6: TetZap70 FoxP3<sup>GFP</sup> mixed chimeras. **B:** Histograms comparing L-selectin (CD62L), HSA and Qa-2 expression on TCR<sup>Hi</sup> CD5<sup>Hi</sup> CD4<sup>+</sup> SP CD25<sup>Hi</sup> CD44<sup>Int</sup> T<sub>reg</sub> from the CD45.1<sup>+</sup> Bl/6 compartment (red) and CD45.1<sup>-</sup> TetZap70 FoxP3<sup>GFP</sup> compartment (blue) of d13 dox fed Bl/6 CD45.1: TetZap70 FoxP3<sup>GFP</sup> mixed chimeras.

3.5

PBS

IL-2 blockade

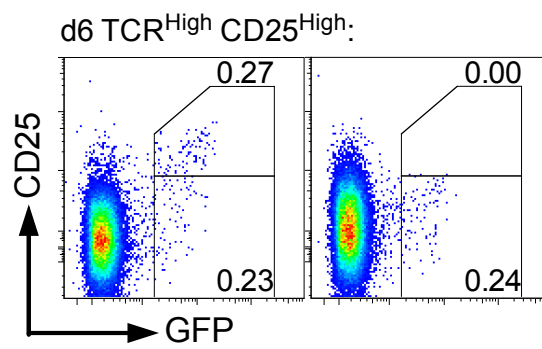
A



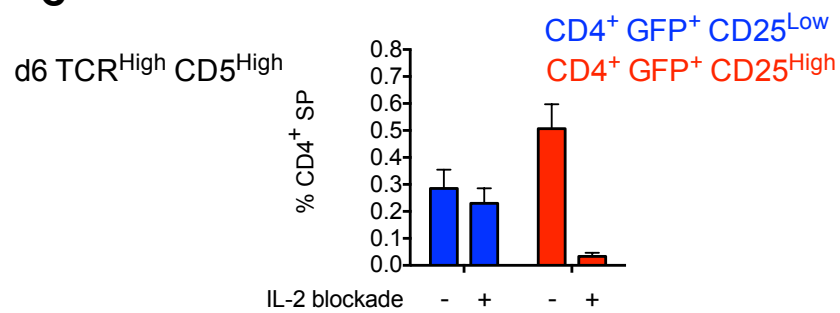
PBS

IL-2 blockade

B



C





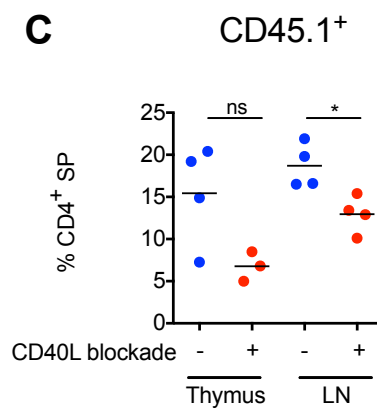
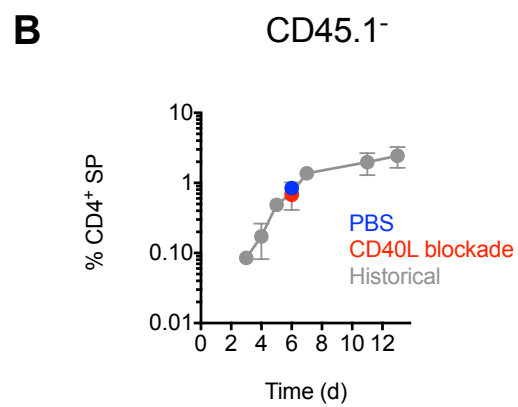
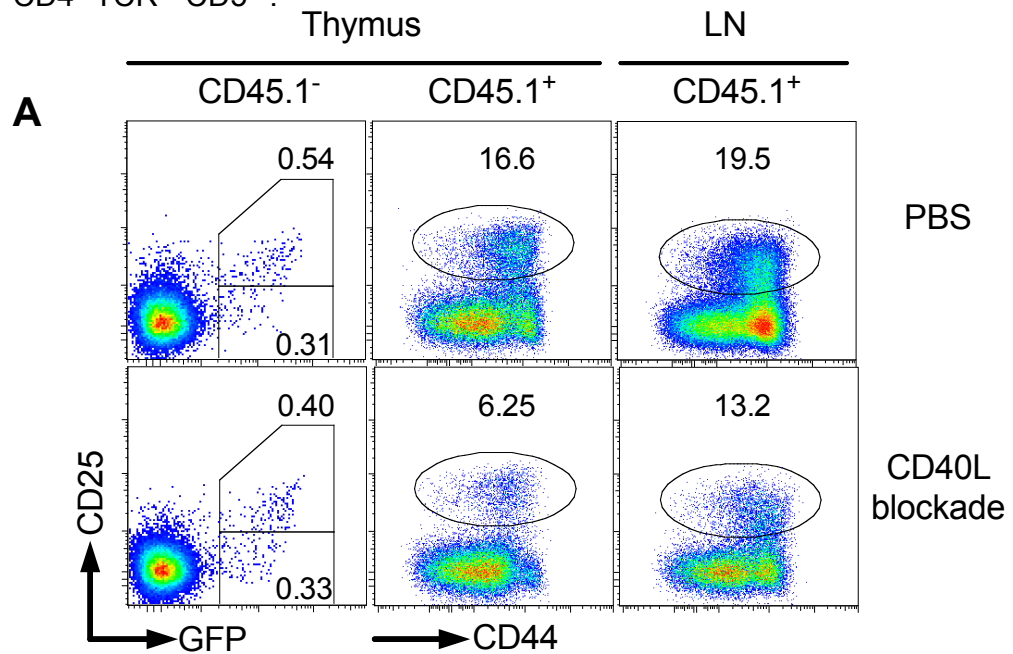
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**Figure 3.5 Reduced CD25 expression on mixed chimeras treated with an IL-2 blockade**

Rag1<sup>-/-</sup> mice were irradiated and injected with 3M T-cell depleted B1/6 CD45.1 bone marrow cells along with 3M TetZap70 FoxP3<sup>GFP</sup> bone marrow cells in a single iv injection. Bone marrow was harvested, T-cell depleted and injected as described in materials and methods. The resulting Chimeras were left for 6 weeks to reconstitute before being treated with 1mg of an IL-2 blocking antibody along with B1/6 FoxP3<sup>GFP</sup> controls via a single ip injection (0.5mg JES6-5H4, 0.5mg S4B6). The following day chimeras were fed dox, receiving additional doses of blocking antibody or PBS, along with B1/6 controls, every 2 days until day 6 when the thymus was analysed by FACs. **A:** FACs plots comparing GFP and HSA expression on TCR<sup>Hi</sup> CD5<sup>Hi</sup> CD4<sup>+</sup> SPs from B1/6 FoxP3<sup>GFP</sup> mice treated with an IL-2 blocking antibody (right) or PBS (left). **B:** FACs plots comparing CD25 and GFP expression on d6 TCR<sup>Hi</sup> CD5<sup>Hi</sup> CD4<sup>+</sup> SP thymocytes from PBS treated (left) and IL-2 blocked (right) B1/6 CD45.1:TetZap70 FoxP3<sup>GFP</sup> mixed chimeras. **C:** Bar charts showing d6 TCR<sup>Hi</sup> CD5<sup>Hi</sup> CD4<sup>+</sup> SP GFP<sup>+</sup> thymocyte frequency, with or without IL-2 blockade. Thymocytes have then been further subdivided depending on CD25 expression, CD25<sup>High</sup> (red) or CD25<sup>Low</sup> (blue) Frequencies displayed as a percentage of TCR<sup>Hi</sup> CD5<sup>Hi</sup> CD4<sup>+</sup> SPs.

### 3.6

CD4<sup>+</sup> TCR<sup>Hi</sup> CD5<sup>Hi</sup>:



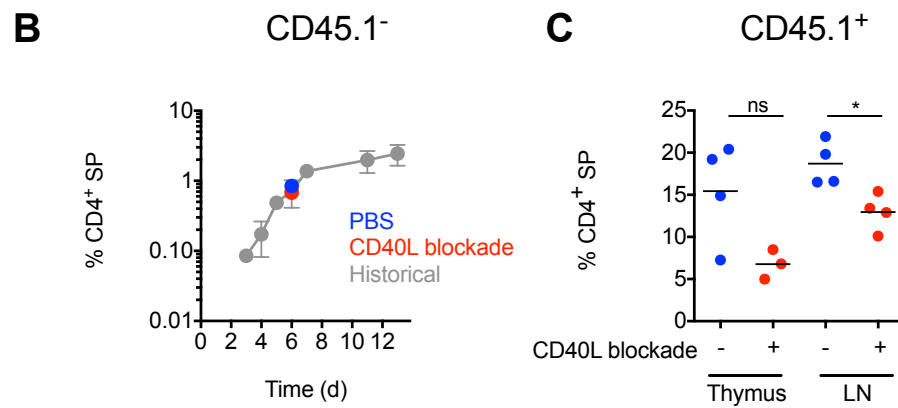
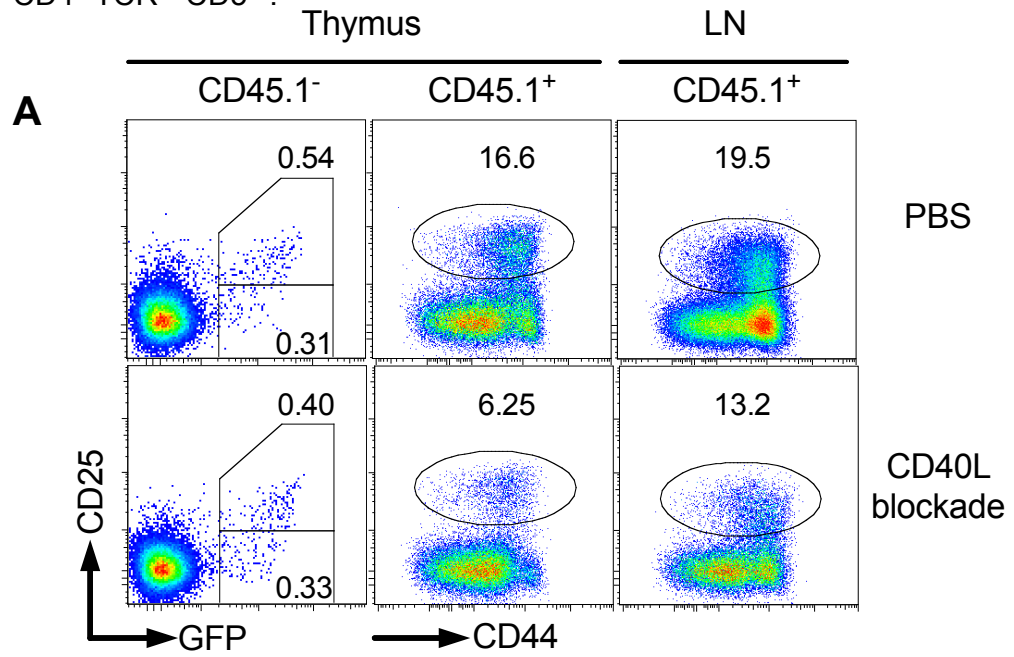
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### Figure 3.6 Normal T<sub>reg</sub> frequencies in CD40L blocked mixed chimeras

Rag1<sup>-/-</sup> mice were irradiated and injected with 3M T-cell depleted B1/6 CD45.1 bone marrow cells along with 3M TetZap70 FoxP3<sup>GFP</sup> bone marrow cells in a single iv injection. Bone marrow was harvested, T-cell depleted and injected as described in materials and methods. The resulting Chimeras were left for 6 weeks to reconstitute before being treated with 0.5 mg of a CD40L blocking antibody via a single ip injection (MR1). The following day chimeras were fed dox, receiving additional doses of blocking antibody or PBS every 2 days until day 6 when the thymus and LNs were analysed by FACs. **A:** FACs plots comparing thymocytes GFP and HSA expression (far left) or CD25 and CD44 expression (middle, right) on TCR<sup>Hi</sup> CD5<sup>Hi</sup> CD4<sup>+</sup> SPs from B1/6 CD45.1: TetZap70 FoxP3<sup>GFP</sup> mice treated with PBS (top) or a CD40L blocking antibody (bottom). **B:** Line graph comparing CD45.1<sup>-</sup> TCR<sup>Hi</sup> CD5<sup>Hi</sup> CD4<sup>+</sup> SP GFP<sup>+</sup> thymocyte frequency from B1/6 CD45.1 : TetZap70 FoxP3<sup>GFP</sup> mixed chimeras treated with PBS (blue) or CD40L blockade (red) compared to historical B1/6 CD45.1 : TetZap70 FoxP3<sup>GFP</sup> mixed chimera time course data. Frequencies expressed as a percentage of TCR<sup>Hi</sup> CD5<sup>Hi</sup> CD4<sup>+</sup> SP cells. **C:** CD45.1<sup>+</sup> TCR<sup>Hi</sup> CD5<sup>Hi</sup> CD4<sup>+</sup> SP CD25<sup>Hi</sup> CD44<sup>Int</sup> frequency in B1/6 CD45.1 : TetZap70 FoxP3<sup>GFP</sup> mixed chimera thymus (left) or LN (right) treated with PBS (blue) or CD40L blockade (red). Frequencies expressed as a percentage of TCR<sup>Hi</sup> CD5<sup>Hi</sup> CD4<sup>+</sup> SP cells.

### 3.6

CD4<sup>+</sup> TCR<sup>Hi</sup> CD5<sup>Hi</sup>:



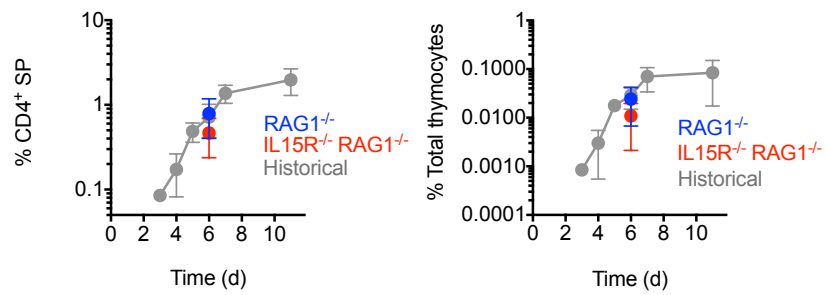
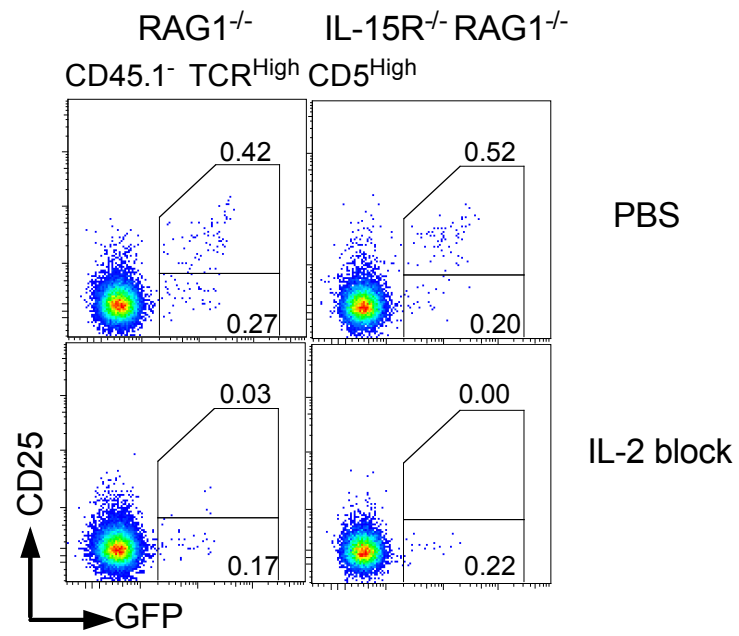
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### Figure 3.6 Normal T<sub>reg</sub> frequencies in CD40L blocked mixed chimeras

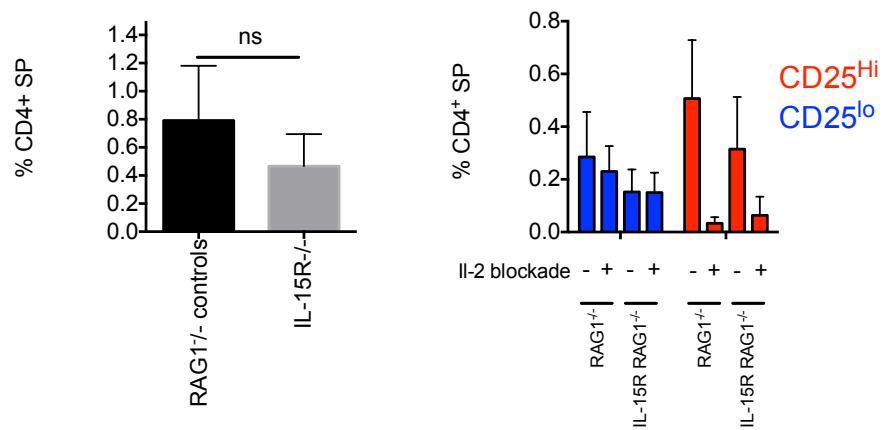
Rag1<sup>-/-</sup> mice were irradiated and injected with 3M T-cell depleted B1/6 CD45.1 bone marrow cells along with 3M TetZap70 FoxP3<sup>GFP</sup> bone marrow cells in a single iv injection. Bone marrow was harvested, T-cell depleted and injected as described in materials and methods. The resulting Chimeras were left for 6 weeks to reconstitute before being treated with 0.5 mg of a CD40L blocking antibody via a single ip injection (MR1). The following day chimeras were fed dox, receiving additional doses of blocking antibody or PBS every 2 days until day 6 when the thymus and LNs were analysed by FACs. **A:** FACs plots comparing thymocytes GFP and HSA expression (far left) or CD25 and CD44 expression (middle, right) on TCR<sup>Hi</sup> CD5<sup>Hi</sup> CD4<sup>+</sup> SPs from B1/6 CD45.1: TetZap70 FoxP3<sup>GFP</sup> mice treated with PBS (top) or a CD40L blocking antibody (bottom). **B:** Line graph comparing CD45.1<sup>-</sup> TCR<sup>Hi</sup> CD5<sup>Hi</sup> CD4<sup>+</sup> SP GFP<sup>+</sup> thymocyte frequency from B1/6 CD45.1 : TetZap70 FoxP3<sup>GFP</sup> mixed chimeras treated with PBS (blue) or CD40L blockade (red) compared to historical B1/6 CD45.1 : TetZap70 FoxP3<sup>GFP</sup> mixed chimera time course data. Frequencies expressed as a percentage of TCR<sup>Hi</sup> CD5<sup>Hi</sup> CD4<sup>+</sup> SP cells. **C:** CD45.1<sup>+</sup> TCR<sup>Hi</sup> CD5<sup>Hi</sup> CD4<sup>+</sup> SP CD25<sup>Hi</sup> CD44<sup>Int</sup> frequency in B1/6 CD45.1 : TetZap70 FoxP3<sup>GFP</sup> mixed chimera thymus (left) or LN (right) treated with PBS (blue) or CD40L blockade (red). Frequencies expressed as a percentage of TCR<sup>Hi</sup> CD5<sup>Hi</sup> CD4<sup>+</sup> SP cells.

### 3.7

**A**



**C**



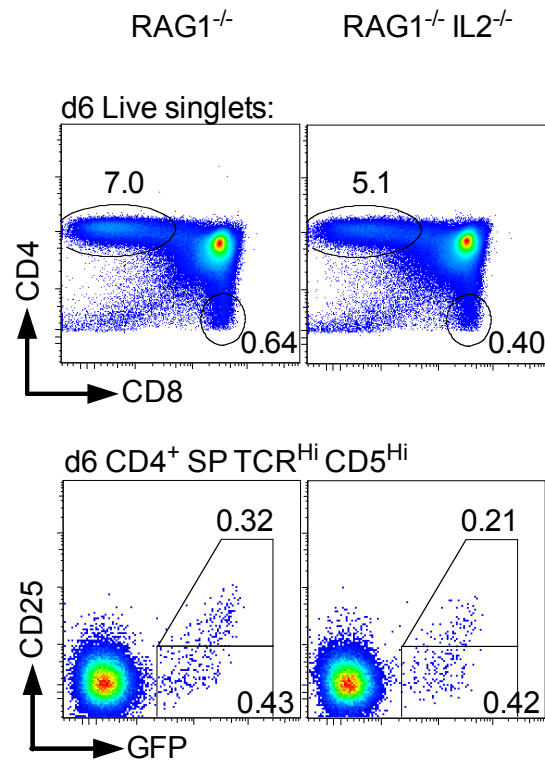
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### Figure 3.7 Characterisation of IL-15R Rag1<sup>-/-</sup> host mixed chimeras

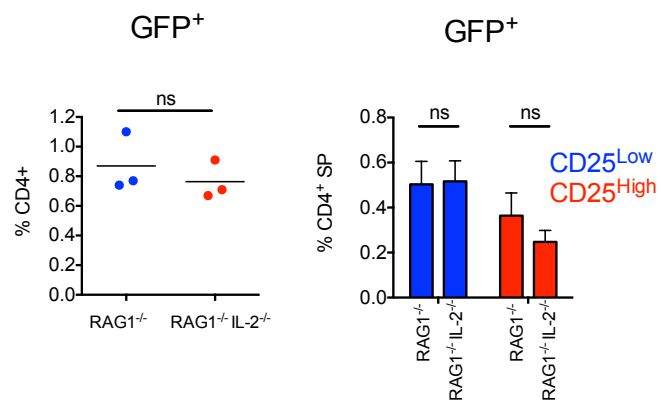
IL-15R<sup>-/-</sup> Rag1<sup>-/-</sup> or Rag1<sup>-/-</sup> mice were irradiated and injected with 3M T-cell depleted Bl/6 CD45.1 bone marrow cells along with 3M TetZap70 FoxP3<sup>GFP</sup> bone marrow cells in a single iv injection. Bone marrow was harvested, T-cell depleted and injected as described in materials and methods. The resulting Chimeras were left for 6 weeks to reconstitute before being treated with either PBS or 1mg of an IL-2 blocking antibody (0.5mg JES6-5H4 + 0.5mg S4B6) via a single ip injection (MR1). The following day chimeras were fed dox, receiving additional doses of blocking antibody or PBS every 2 days until day 6 when the thymocytes were analysed by FACs. **A:** FACs plots comparing thymocytes GFP and HSA expression on CD45.1<sup>-</sup> TCR<sup>Hi</sup> CD5<sup>Hi</sup> CD4<sup>+</sup> SPs from Bl/6 CD45.1: TetZap70 FoxP3<sup>GFP</sup> IL-15R<sup>-/-</sup> Rag1<sup>-/-</sup> (Right panel) or Rag1<sup>-/-</sup> (left panel) host mixed chimeras. Mice treated with PBS (top) or an IL-2 blocking antibody (bottom). **B:** Line graph comparing CD45.1<sup>-</sup> TCR<sup>Hi</sup> CD5<sup>Hi</sup> CD4<sup>+</sup> SP GFP<sup>+</sup> thymocyte frequency from Bl/6 CD45.1 : TetZap70 FoxP3<sup>GFP</sup> mixed chimeras in IL-15R<sup>-/-</sup> Rag1<sup>-/-</sup> (red) or Rag1<sup>-/-</sup> (blue) hosts. Historical Bl/6 CD45.1 : TetZap70 FoxP3<sup>GFP</sup> mixed chimera Rag1<sup>-/-</sup> hosts in grey. Frequencies expressed as a percentage of TCR<sup>Hi</sup> CD5<sup>Hi</sup> CD4<sup>+</sup> SP cells (left) or total thymocytes (right). **C:** Bar charts showing CD45.1<sup>-</sup> TCR<sup>Hi</sup> CD5<sup>Hi</sup> CD4<sup>+</sup> SP GFP<sup>+</sup> thymocyte frequency in IL-15R<sup>-/-</sup> Rag1<sup>-/-</sup> or Rag1<sup>-/-</sup> chimeras (left) with or without IL-2 blockade (right). Thymocytes have then been further subdivided depending on CD25 expression (right) into CD25<sup>High</sup> (red) or CD25<sup>Low</sup> (blue) Frequencies displayed as a percentage of TCR<sup>Hi</sup> CD5<sup>Hi</sup> CD4<sup>+</sup> SPs.

3.8

A



B



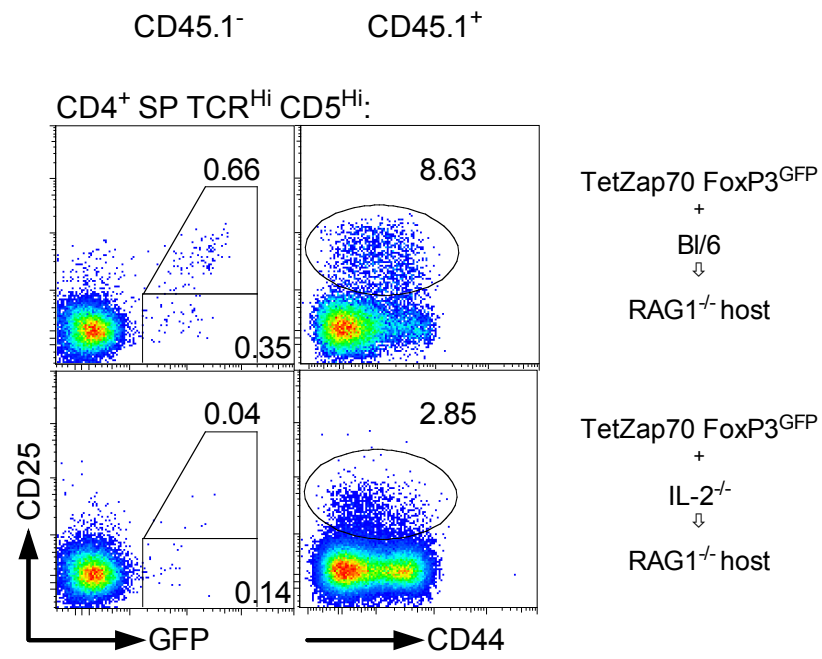


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### Figure 3.8 Non-stromal source of IL-2 required for CD25<sup>Hi</sup> T<sub>reg</sub> induction

IL-2<sup>-/-</sup> Rag1<sup>-/-</sup> or Rag1<sup>-/-</sup> mice were irradiated and injected with 3M TetZap70 FoxP3<sup>GFP</sup> bone marrow cells in a single iv injection. Bone marrow was harvested and injected as described in materials and methods. The resulting chimeras were left for 6 weeks to reconstitute before being fed dox until day 6 when the thymocytes were analysed by FACs. **A:** FACs plots comparing CD4 and CD8 expression on live singlets from IL-2<sup>-/-</sup> Rag1<sup>-/-</sup> (top right) or Rag1<sup>-/-</sup> (top left) host TetZap70 FoxP3<sup>GFP</sup> chimeras. (bottom panel) FACs plots comparing CD25 and GFP expression on TCR<sup>Hi</sup> CD5<sup>Hi</sup> CD4<sup>+</sup> SP GFP<sup>+</sup> thymocytes in IL-2<sup>-/-</sup> Rag1<sup>-/-</sup> (bottom right) or Rag1<sup>-/-</sup> (bottom left) host TetZap70 FoxP3<sup>GFP</sup> chimeras. **B:** Bar charts showing CD45.1<sup>-</sup> TCR<sup>Hi</sup> CD5<sup>Hi</sup> CD4<sup>+</sup> SP GFP<sup>+</sup> thymocyte frequency in IL-2<sup>-/-</sup> Rag1<sup>-/-</sup> or Rag1<sup>-/-</sup> chimeras (left). Thymocytes have then been further subdivided depending on CD25 expression (right) into CD25<sup>High</sup> (red) or CD25<sup>Low</sup> (blue) Frequencies displayed as a percentage of TCR<sup>Hi</sup> CD5<sup>Hi</sup> CD4<sup>+</sup> SPs.

### 3.9

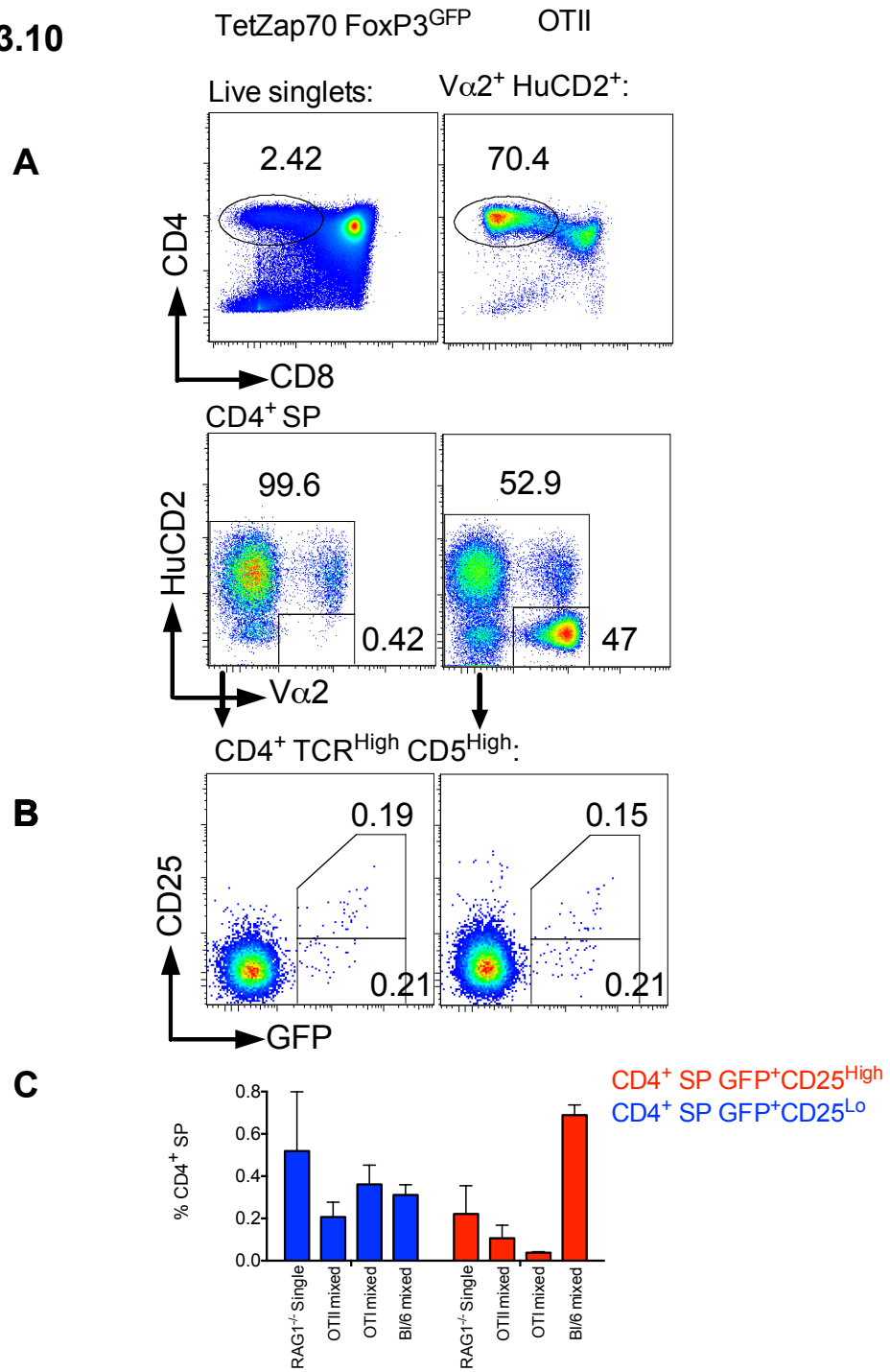


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**Figure 3.9 Hematopoietic source of IL-2 required for CD25<sup>Hi</sup> T<sub>reg</sub> induction.**

Rag1<sup>-/-</sup> mice were irradiated and injected with 3M TetZap70 FoxP3<sup>GFP</sup> bone marrow cells and either 3M Bl/6 CD45.1 (Bl/6 CD45.1:TetZap70 FoxP3<sup>GFP</sup>) or 3M IL-2<sup>-/-</sup> bone marrow cells (IL-2<sup>-/-</sup>:TetZap70 FoxP3<sup>GFP</sup>) in a single iv injection. Bone marrow was harvested, T-cell depleted and injected as described in materials and methods. The resulting chimeras were left for 6 weeks to reconstitute before being fed dox until day 6 when the thymocytes were analysed by FACs. FACs plots comparing GFP and CD25 expression (left) on CD45.1<sup>-</sup> TCR<sup>Hi</sup> CD5<sup>Hi</sup> CD4<sup>+</sup> SP thymocytes from IL-2<sup>-/-</sup>:TetZap70 FoxP3<sup>GFP</sup> mixed chimeras (bottom) or Bl/6 CD45.1:TetZap70 FoxP3<sup>GFP</sup> mixed chimeras (top). FACs plots comparing CD44 and CD25 expression (right panel) on CD45.1<sup>+</sup> TCR<sup>Hi</sup> CD5<sup>Hi</sup> CD4<sup>+</sup> SP thymocytes from IL-2<sup>-/-</sup>:TetZap70 FoxP3<sup>GFP</sup> mixed chimeras (bottom) or Bl/6 CD45.1:TetZap70 FoxP3<sup>GFP</sup> mixed chimeras (top).

### 3.10

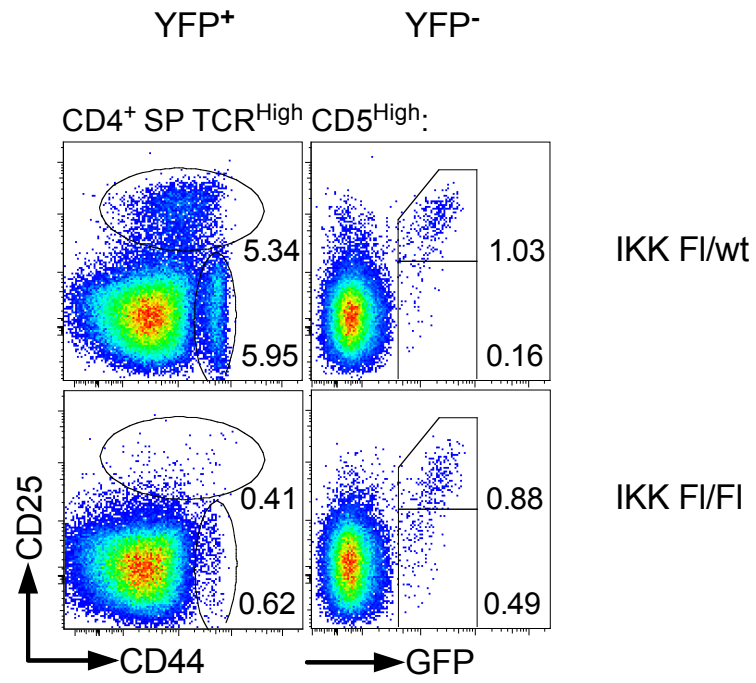


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### Figure 3.10 TCR transgenic TetZap70 FoxP3<sup>GFP</sup> mixed chimeras

Rag1<sup>-/-</sup> mice were irradiated and injected with 3M TetZap70 FoxP3<sup>GFP</sup> bone marrow cells and 3M OTII or OTI bone marrow cells (OTII/OTI:TetZap70 FoxP3<sup>GFP</sup>) contained within a single iv injection. Irradiated Rag1<sup>-/-</sup> mice were also injected with 3M TetZap70 FoxP3<sup>GFP</sup> only, as controls. Bone marrow was harvested and injected as described in materials and methods. The resulting chimeras were left for 6 weeks to reconstitute before being fed dox until day 6 when the thymocytes were analysed by FACs. **A:** FACs plots comparing CD4 and CD8 expression (top panel) on live singlets (left) or V $\alpha$ 2<sup>+</sup> live singlets (right) from OTII/OTI:TetZap70 FoxP3<sup>GFP</sup> chimeras (right) and TetZap70 FoxP3<sup>GFP</sup> chimers (left). (bottom panel) FACs plots comparing HuCD2 and V $\alpha$ 2 expression on TCR<sup>Hi</sup> CD5<sup>+</sup> CD4<sup>+</sup> SPs for OTII/OTI:TetZap70 FoxP3<sup>GFP</sup> chimeras (right) and TetZap70 FoxP3<sup>GFP</sup> chimers (left). **B:** FACs plots comparing CD25 and GFP on TCR<sup>Hi</sup> CD5<sup>+</sup> CD4<sup>+</sup> SP V $\alpha$ 2<sup>-</sup> cells from OTII/OTI:TetZap70 FoxP3<sup>GFP</sup> chimeras (right) and TetZap70 FoxP3<sup>GFP</sup> chimers (left). **C:** Bar chart showing frequencies of TCR<sup>Hi</sup> CD5<sup>+</sup> CD4<sup>+</sup> SP V $\alpha$ 2<sup>-</sup> GFP<sup>+</sup> CD25<sup>Hi</sup> (red) and CD25<sup>Lo</sup> (Blue) cells from OTII:TetZap70 FoxP3<sup>GFP</sup>, OTI:TetZap70 FoxP3<sup>GFP</sup> and TetZap70 FoxP3<sup>GFP</sup> chimeras, as a percentage of TCR<sup>Hi</sup> CD5<sup>+</sup> CD4<sup>+</sup> SP V $\alpha$ 2<sup>-</sup> cells.

### 3.11



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**Figure 3.11 IKK2<sup>FI/FI</sup> CD4 Cre R26:TetZap70 FoxP3<sup>GFP</sup> Rag1<sup>-/-</sup> host chimeras**

Rag1<sup>-/-</sup> mice were irradiated and injected with 3M TetZap70 FoxP3<sup>GFP</sup> bone marrow cells and 3M IKK2<sup>FI/FI</sup> CD4 Cre R26 bone marrow cells (IKK2<sup>FI/FI</sup> CD4 Cre R26:TetZap70 FoxP3<sup>GFP</sup>) or IKK2<sup>FI/WT</sup> CD4 Cre R26 bone marrow cells (IKK2<sup>FI/WT</sup> CD4 Cre R26:TetZap70 FoxP3<sup>GFP</sup>) contained within a single iv injection. Bone marrow was harvested and injected as described in materials and methods. The resulting chimeras were left for 6 weeks to reconstitute before being fed dox until day 10 when the thymocytes were analysed by FACs. FACs plots comparing CD44 and CD25 expression (left panel) on TCR<sup>Hi</sup> CD5<sup>+</sup> CD4<sup>+</sup> SP YFP<sup>+</sup> cells from IKK2<sup>FI/FI</sup> CD4 Cre R26:TetZap70 FoxP3<sup>GFP</sup> mixed chimeras (bottom) or IKK2<sup>FI/WT</sup> CD4 Cre R26:TetZap70 FoxP3<sup>GFP</sup> mixed chimeras (top). FACs plots comparing GFP and CD25 expression (right panel) on TCR<sup>Hi</sup> CD5<sup>+</sup> CD4<sup>+</sup> SP YFP<sup>-</sup> cells from IKK2<sup>FI/FI</sup> CD4 Cre R26:TetZap70 FoxP3<sup>GFP</sup> mixed chimeras (bottom) or IKK2<sup>FI/WT</sup> CD4 Cre R26:TetZap70 FoxP3<sup>GFP</sup> mixed chimeras (top).

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## Discussion

IL-2 has long been thought to play a central role in T<sub>reg</sub> development and homeostasis. However its role during thymic T<sub>reg</sub> development remains only partially understood. In this study we confirm IL-2 dependant generation of CD4<sup>+</sup> SP CD25<sup>Hi</sup> GFP<sup>+</sup> T<sub>reg</sub>. TetZap70 FoxP3<sup>GFP</sup> single chimeras appeared to lack the ability to drive efficient development of CD25<sup>Hi</sup> GFP<sup>+</sup> T<sub>reg</sub>. Enhanced development in Bl/6 : TetZap70 FoxP3<sup>GFP</sup> mixed chimeras appeared to be due to the presence of an IL-2 producing cellular compartment which was reduced in TetZap70 FoxP3<sup>GFP</sup> single chimeras. We speculate a haematopoietic source for this IL-2-producing cell type, which is most likely derived from the Bl/6 WT bone marrow. IL-2 is predominantly produced by activated CD4<sup>+</sup> SPs and to a lesser extent CD8<sup>+</sup> SP T-cells. Activated DCs, NK and NKT cells may also produce IL-2, however the biological relevance of IL-2 production by these cell types remains incompletely understood (Reviewed in Malek 2008). It has been shown that activated CD4<sup>+</sup> SPs are the primary producers of IL-2 in the immune system (Naramura et al. 1998). This is inconsistent with our observations made in TetZap70 FoxP3<sup>GFP</sup> mixed chimeras using TCR Tg partner bone marrow. MHCII restricted CD4<sup>+</sup> SP OTII TCR transgenic T-cells were incapable of supporting CD4<sup>+</sup> SP CD25<sup>Hi</sup> GFP<sup>+</sup> T<sub>reg</sub> development. As IL-2 is secreted by T-cells upon activation (Yang-snyder & Ellen V Rothenberg 1998), the absence of cognate ligand in the OTII chimeras would have lead to no T-cell activation and



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IL-2 production. Such a scenario may suggest that peripherally derived  $T_{\text{mem}}$  cells could be the primary source of thymic IL-2, which they secrete after recirculating back into the thymus. We previously demonstrated the recirculation of mature T-cells into the thymus in chapter 1. Mature  $CD4^+$  SP and  $CD8^+$  SP T-cells were shown to recirculate back to the thymus of TetZap70 FoxP3<sup>GFP</sup> mice. However these did not support efficient  $CD4^+$  SP  $CD25^{\text{Hi}}$  GFP<sup>+</sup>  $T_{\text{reg}}$  generation, casting doubt over the role played by mature recirculating cells as a source of IL-2 in the thymus. Further doubt over a role for recirculating activated T-cells comes from IKK2 Fl/Fl  $CD4\text{-Cre}$  R26 TetZap70 FoxP3<sup>GFP</sup> mixed chimeras which were able to support efficient  $CD4^+$  SP  $CD25^{\text{Hi}}$  GFP<sup>+</sup>  $T_{\text{reg}}$  development in the absence of an activated memory T-cell compartment. Taken together it seems unlikely that IL-2 from a recirculant activated T memory cell population is driving  $CD4^+$  SP  $CD25^{\text{Hi}}$  GFP<sup>+</sup>  $T_{\text{reg}}$  generation. However a further explanation for the OTII mixed chimeras inability to support  $CD4^+$  SP  $CD25^{\text{Hi}}$  GFP<sup>+</sup>  $T_{\text{reg}}$  generation is sub-optimal TCR stimulation during thymic selection. As T-cells in the thymus of TCR transgenic mixed chimeras are selected on self-antigens it remains a possibility that competition by identical TCR clones for similar epitopes may have lead to sub-optimal TCR stimulation during selection. This scenario could suggest a role for positive and negative selection as the main source of thymic IL-2. This is supported by data from IKK2 Fl/Fl  $CD4\text{-Cre}$  R26 TetZap70 FoxP3<sup>GFP</sup> mixed chimeras which contain naïve polyclonal  $CD4^+$  SPs and were able to support  $CD4^+$  SP  $CD25^{\text{Hi}}$  GFP<sup>+</sup>  $T_{\text{reg}}$  generation. T-cells in these mice may have received stronger selection signals due to less competition for thymic epitopes, leading to increased T-cell activation and IL-2 secretion. Previous

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studies have also found an important role for IL-2 in generating CD25<sup>Hi</sup> FoxP3<sup>+</sup> T<sub>reg</sub>, however to our knowledge no work to date has focussed on the source of this thymic IL-2. Previous studies suggest that thymic IL-2 is most likely to be T-cell derived, as bone marrow chimeras using IL-2 deficient donors and WT hosts were phenotypically similar to that of IL-2 deficient mice (Tai et al. 2005). IL-2 was also shown to exert its effect in a paracrine manner as IL-2 deficient and sufficient cells showed equal expression of CD25. Furthermore it has been shown that T-cells undergoing apoptosis can release IL-2 (Bassiri & Carding 2001). These studies would seem to support our suggestion of a role for T-cells undergoing negative selection as a source of paracrine IL-2 in the thymus in order to drive efficient CD4<sup>+</sup> SP CD25<sup>Hi</sup> GFP<sup>+</sup> T<sub>reg</sub> generation.

Most studies focus on IL-2 as the primary signalling cue which initiates FoxP3 induction. Previous studies have shown that the first detectable FoxP3<sup>+</sup> cells in neonates have high surface expression of CD25 (Fontenot, Rasmussen, Williams, et al. 2005)(Cheng et al. 2013). This has been put forward as support for T<sub>reg</sub> developing via a CD25<sup>Hi</sup> FoxP3<sup>-</sup> precursor population in an IL-2 dependant manner. However we show IL-2 independent FoxP3 induction in mixed chimeras treated with an IL-2 blocking mAb. In these mice we see T<sub>reg</sub> developing with low expression of CD25. IL-2 producing T-cells can be found in neonates from day 14 of gestation (Yang-Snyder & E V Rothenberg 1998). Neonatal development may therefore be analogous to that of the mixed chimeras, in which IL-2 producing cells cause T<sub>reg</sub> to rapidly increase CD25 expression without necessarily directly inducing FoxP3. We did however

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observe decreased  $T_{reg}$  frequencies in TetZap70 FoxP3<sup>GFP</sup> mixed chimeras which received the mAb blockade. It therefore remains a possibility that an IL-2 dependant and independent routes of development exist. CD25<sup>Hi</sup> FoxP3<sup>-</sup> cells may be driven to express FoxP3 by IL-2, becoming CD25<sup>Hi</sup> FoxP3<sup>+</sup> cells. While CD25<sup>Lo</sup> FoxP3<sup>+</sup> cells may develop independently of the IL-2 signal. IL-2 blockade in TetZap70 FoxP3<sup>GFP</sup> mixed chimeras may block the IL-2 dependant development and leave the second route of development intact, leading to decreased  $T_{reg}$  frequencies. If true this would further support a 2 step model for IL-2 dependant FoxP3 expression. IL-2 however is not only implicated in FoxP3 induction but suggested to play a crucial role in  $T_{reg}$  expansion and homeostasis (Reviewed in Malek 2008). A recent study has shown that in the absence of IL-2R $\beta$  signalling  $T_{reg}$  develop predominantly CD25<sup>Lo</sup> and express low levels of the proliferation marker Ki67 (Cheng et al. 2013). Reduced  $T_{reg}$  frequencies in mixed chimeras which received the IL-2 mAb blockade could therefore be explained by reduced proliferation of  $T_{reg}$  in the absence of an IL-2 signal. Further to this we observe increased  $T_{reg}$  frequencies in TetZap70 FoxP3<sup>GFP</sup> mice treated with IL-2-mAb complex. Bl/6 : TetZap70 FoxP3<sup>GFP</sup> mixed chimeras contain a hematopoietic source of IL-2 which could support enhanced  $T_{reg}$  proliferation compared to single chimera controls. This proliferative capacity appears to be most likely occurring at later time points when  $T_{reg}$  frequencies are at their highest.

An additional explanation for the increased frequencies of  $T_{reg}$  observed in Bl/6 : TetZap70 FoxP3<sup>GFP</sup> mixed chimeras is the maturation of the thymic stroma. Un-

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induced TetZap70 FoxP3<sup>GFP</sup> mice completely lack a T-cell compartment. Development in these mice is therefore analogous to that of a neonate, in which mature T-cells have not yet developed. A fully mature thymic T-cell compartment has been shown to be crucial for the development of a fully mature thymic microenvironment. Activation of CD40 and RANK by CD40L and RANKL on mature SPs supply signals leading to the maturation of the thymic stromal cells (Akiyama et al. 2008). Lymphotoxin  $\beta$  signals have also been shown to be crucial for the differentiation of involucrin<sup>+</sup> mTECs, a specific subset of terminally differentiated mTECs which are themselves implicated in T<sub>reg</sub> development (White et al. 2010)(Cowan et al. 2013). A fully mature T-cell compartment may therefore be essential to ensure maturation of the thymic stroma and maintenance of central and peripheral tolerance. TetZap70 FoxP3<sup>GFP</sup> mixed chimeras unlike TetZap70 FoxP3<sup>GFP</sup> single chimeras contain a full T-cell compartment. Development in these mice is therefore more analogous to that of development in an adult mouse. We showed increased T<sub>reg</sub> frequencies in TetZap70 FoxP3<sup>GFP</sup> mixed chimeras at later time points compared to single chimera controls. It therefore remains a possibility that in the presence of a fully mature thymic stroma TetZap70 FoxP3<sup>GFP</sup> mixed chimeras are more able to support efficient T<sub>reg</sub> development, leading to increased frequencies. This however fails to account for the increased expression on CD25 on developing T<sub>reg</sub>. Using IL-2<sup>-/-</sup> Rag1<sup>-/-</sup> host chimeras, we show no role for the thymic stroma as a source of IL-2 during development. Although it remains a distinct possibility that important contributions from the thymic stroma exist, it seems likely that the main reason for increased T<sub>reg</sub> frequencies in

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TetZap70 FoxP3<sup>GFP</sup> mixed chimeras is additional proliferation in the presence of IL-2.

We previously suggested a redundancy existed between IL-2 and IL-15 signalling during de novo T<sub>reg</sub> development in TetZap70 FoxP3<sup>GFP</sup> chimeras. In Bl/6 :TetZap70 FoxP3<sup>GFP</sup> mixed chimeras we show a small difference in T<sub>reg</sub> frequencies between IL-15R<sup>-/-</sup> Rag1<sup>-/-</sup> and Rag1<sup>-/-</sup> hosts however this was not statistically significant. This is a much smaller difference than previously observed in TetZap70 FoxP3<sup>GFP</sup> chimeras. Previous studies show normal proportions of T<sub>reg</sub> in IL-15<sup>-/-</sup> and IL-15Rα<sup>-/-</sup> mice (M. A. Burchill et al. 2007) suggesting no role for IL-15 in normal T<sub>reg</sub> development and homeostasis. However one study has shown that IL-2Rβ<sup>-/-</sup> mice contain fewer thymic T<sub>reg</sub> than IL-2<sup>-/-</sup> mice (M. A. Burchill et al. 2007), suggesting contributions by both IL-2 and IL-15 to T<sub>reg</sub> biology. IL-15 and IL-2 both share components of their receptors and activate the STAT5 signalling pathway. Therefore it remains highly likely that some degree of redundancy exists between the two cytokines. This is consistent with the slight contraction we observe in T<sub>reg</sub> frequencies in Bl/6 : TetZap70 FoxP3<sup>GFP</sup> IL-15R<sup>-/-</sup> mixed chimeras. We have suggested that the mixed chimeras contain much higher levels of thymic IL-2 than single chimera controls. Therefore ablating IL-15 signalling in these mice may have less effect. Conversely in the TetZap70 FoxP3<sup>GFP</sup> single chimeras which appear to be lacking in thymic IL-2, ablation of IL-15 signalling leads to an approximate 50% decrease in thymic T<sub>reg</sub>. Our data therefore appears consistent with a redundancy existing between IL-2 and IL-15 where IL-2 plays the dominant role

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in T<sub>reg</sub> development and IL-15 is capable of compensating in situations of low IL-2 concentration. Blockade of IL-2 however in IL-15R<sup>-/-</sup> chimeras does not completely halt T<sub>reg</sub> development altogether, suggesting possible roles for additional factors. Another  $\gamma$ c cytokine, IL-7 has been shown to play a role in T<sub>reg</sub> homeostasis (Latour et al. 2013). However IL-7<sup>-/-</sup> mice contain normal T<sub>reg</sub> frequencies (Latour et al. 2006). It remains a possibility that IL-7 is capable of supporting low levels of T<sub>reg</sub> development in the absence of IL-2 and IL-15, perhaps though promoting survival. But in thymi where IL-2 is abundant, knocking out IL-7 has very little effect. Taken together our data coupled with the current literature suggest an important role for IL-2 in FoxP3<sup>+</sup> CD25<sup>Hi</sup> T<sub>reg</sub> development. IL-2<sup>-/-</sup> mice however still contain approximately 50% of their T<sub>reg</sub> compartment while  $\gamma$ c<sup>-/-</sup> have no FoxP3<sup>+</sup> T<sub>reg</sub> whatsoever (Fontenot, Rasmussen, Gavin & A. Y. Rudensky 2005). It therefore seems likely that multiple  $\gamma$ c cytokines co-operate during T<sub>reg</sub> development.

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## Chapter 6

### Discussion

The overall aim of this study was to investigate the mechanisms regulating the thymic development of  $T_{reg}$ . More specifically we aimed to identify the immediate precursors to  $T_{reg}$  and the differentiation signals required for FoxP3 induction. Where previous studies have attempted this analysis in lymphoreplete animals, we studied the first wave de novo  $T_{reg}$  development without the added complication of recirculant mature populations. This allowed analysis of the timing, phenotype and the signalling cues required for efficient  $T_{reg}$  development. Further to this we were able to identify a requirement for additional cell types in  $T_{reg}$  differentiation, suggesting multiple cell types co-operate in-order to establish peripheral tolerance.

#### **Which thymic populations act as $T_{reg}$ precursors?**

It has been suggested that the identification of a  $T_{reg}$  precursor population would simplify future studies aiming to understand the  $T_{reg}$  differentiation process. Current models for  $T_{reg}$  development suggest that differentiation occurs via a  $CD25^{Hi}$  FoxP3<sup>-</sup> precursor population. In support of this we show that upon sorting  $CD25^{Lo}$  GFP<sup>+</sup>,  $CD25^{Hi}$  GFP<sup>-</sup> cells, the  $CD25^{Hi}$  GFP<sup>-</sup> cells display the most efficient FoxP3 induction when stimulated with IL-2. However gating on GITR (a marker which is elevated in  $T_{reg}$  precursors) dramatically reduces efficiency of FoxP3 induction in these cells, making  $CD25^{Lo}$  GFP<sup>+</sup> cells the most efficient precursors to  $CD25^{Hi}$  GFP<sup>+</sup>  $T_{reg}$ . Previous studies fail to investigate the

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potential of these cells as CD25<sup>Hi</sup> FoxP3<sup>+</sup> T<sub>reg</sub> precursors, instead solely focussing on CD25<sup>Hi</sup> FoxP3<sup>-</sup> cells. This observation suggests CD25<sup>Hi</sup> GFP<sup>+</sup> T<sub>reg</sub> may develop via a CD25<sup>Lo</sup> FoxP3<sup>+</sup> precursor as well as via the previously described CD25<sup>Hi</sup> FoxP3<sup>-</sup> subset. Further to this, in time courses with TetZap70 FoxP3<sup>GFP</sup> mice, T<sub>reg</sub> are detectable on day 4. These T<sub>reg</sub> initially express low levels of CD25, appearing to increase CD25 and FoxP3 expression levels at later time points. In addition to this we observe CD25<sup>Hi</sup> GFP<sup>-</sup> 'precursors' have high HSA expression levels that are progressively lower on CD25<sup>Lo</sup> GFP<sup>+</sup> and CD25<sup>Hi</sup> GFP<sup>+</sup> cells respectively. This could imply that CD25<sup>Lo</sup> GFP<sup>+</sup> cells are the direct precursor to CD25<sup>Hi</sup> GFP<sup>+</sup> T<sub>reg</sub>. Taking advantage of Nur77<sup>GFP</sup> mice we show enrichment for T<sub>reg</sub> precursors in GITR<sup>Hi</sup> Nur77<sup>Hi</sup> cells. When combined with TetZap70 mice, the resulting TetZap70 Nur77<sup>GFP</sup> thymocytes have identical Nur77<sup>GFP</sup> expression levels on CD25<sup>Lo</sup> GFP<sup>+</sup> and CD25<sup>Hi</sup> GFP<sup>+</sup> cells, suggesting similar TCR signalling processes are involved in generating both populations. In contrast, CD25<sup>Hi</sup> GFP<sup>-</sup> cells express higher levels of GFP relative to both populations implying they may represent a distinct TCR repertoire, having experienced strong selection signals. To summarise, this study presents data suggesting that T<sub>reg</sub> develop via a CD25<sup>Lo</sup> GFP<sup>+</sup> precursor. It does not however exclude the possibility that development is also occurring via CD25<sup>Hi</sup> GFP<sup>-</sup> cells as described by the 2 step model (Chan-Wang Joaquim Lio & C.-S. Hsieh 2008).

Although our data suggests a strong possibility that T<sub>reg</sub> differentiate from CD25<sup>Lo</sup> FoxP3<sup>+</sup> precursors, it remains entirely possible that differentiation



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occurs via both CD25<sup>Lo</sup> and CD25<sup>Hi</sup> routes. If true this would cast doubt on the role played by IL-2 in the initiation of FoxP3 induction. CD25 expression may instead only correlate with induction of FoxP3, with cells receiving a stronger selection signal simply expressing CD25 before FoxP3, while weaker selecting cells begin FoxP3 expression before CD25. In support of this we observe increased Nur77<sup>GFP</sup> expression on CD25<sup>Hi</sup> FoxP3<sup>-</sup> cells relative to CD25<sup>Lo</sup> FoxP3<sup>+</sup> cells. These two precursor populations may therefore represent 2 alternate routes, with the same ultimate development fate. Alternatively each population may represent two distinct routes of development, by two different cell types. The CD25<sup>Lo</sup> FoxP3<sup>+</sup> population, not having expressed CD25 and therefore gained IL-2 responsiveness, may represent an immature intermediate still auditioning to become a fully committed T<sub>reg</sub>. IL-2 is known to be essential for maintenance of T<sub>reg</sub> homeostasis, CD25<sup>Lo</sup> FoxP3<sup>+</sup> cells may therefore be prone to losing FoxP3 expression and becoming 'exT<sub>regs</sub>'. In support of this we observed the lower levels of FoxP3 expression in CD25<sup>Lo</sup> FoxP3<sup>+</sup> T<sub>reg</sub> than the mature CD25<sup>Hi</sup> subset. Further to this the Rudensky lab has reported an increase in exT<sub>reg</sub> in mice which receive an IL-2 antibody blockade, a treatment which we shown to decrease CD25 expression on developing T<sub>reg</sub> (Rubtsov et al. 2010).

In order to confirm development of CD25<sup>Hi</sup> FoxP3<sup>+</sup> T<sub>reg</sub> is occurring via CD25<sup>Lo</sup> FoxP3<sup>+</sup> cells, future studies should focus on sorting sufficient numbers of the precursor populations that can be followed following intrathymic injection. Analysis of the thymus 2-3 days post injection would allow us to follow both the

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survival and developmental fate of the various populations. With analysis of this kind it would be possible to draw conclusions regarding precursor-product relationships between the CD25<sup>Lo</sup> FoxP3<sup>+</sup>, CD25<sup>Hi</sup> FoxP3<sup>-</sup> and CD25<sup>Hi</sup> FoxP3<sup>+</sup> cells. If this were to be carried out using wt mice care would have to be taken on sorting immature de novo generated cells, while avoiding more mature recirculating populations. Recirculating T-cells are likely to be a heterogeneous population containing both iT<sub>reg</sub>, nT<sub>reg</sub> amongst other T-cell populations, possibly complicating the interpretation of results. It may also be interesting to further study the methylation status of the FoxP3 locus in the various precursor populations. This would reveal crucial information about the ability of the various populations to both express FoxP3 and maintain expression.

### **What are the signalling cues required for FoxP3 expression?**

Understanding the molecular cues which lead to FoxP3 expression will allow greater insights into both the thymic differentiation of T<sub>reg</sub> and how expression of the FoxP3 gene is regulated. Understanding this process remains a continuing focus for researchers, with an aim to identifying therapeutic targets for autoimmune diseases. In this study, using TetZap70 FoxP3<sup>GFP</sup> mice pulsed with methacycline, we present evidence describing a requirement for a short early TCR signal during thymic selection for T<sub>reg</sub>. Further to this we describe an essential role for CD28 co-stimulatory signalling. When treating TetZap70 FoxP3<sup>GFP</sup> chimeras with an anti-CD28 blocking antibody, T<sub>reg</sub> development is almost completely absent. This data would suggest that an early selection signal involving both TCR and CD28 co-stimulation is needed for FoxP3

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induction. Although it appears an early TCR signal is sufficient for development, we can only speculate as to the temporal requirement for CD28. Indeed further work should aim to confirm whether the role for CD28 in re-enforcing TCR induced T<sub>reg</sub> selection or if CD28 activates crucial pathways in parallel of TCR signalling.

Although we appear to show early signalling is sufficient to support T<sub>reg</sub> induction, the precise nature of this signal remains poorly understood. It remains unclear if the signal received by DP thymocytes during positive selection is enough to trigger a commitment to the T<sub>reg</sub> lineage or in fact multiple rounds of continuous TCR-peptide interactions support development. Observations made during experiments in which TetZap70 FoxP3<sup>GFP</sup> mice were pulsed with methacycline appears to suggest that an early signal is enough to signal development into T<sub>reg</sub>. Observations in these experiments also showed a gradual enrichment of T<sub>reg</sub> compared to conventional T-cell populations. This would appear to suggest that T<sub>reg</sub> are better equipped to survive in the absence of TCR signalling, perhaps due to increased expression of anti-apoptotic proteins many of which are the target of FoxP3. This may reflect a requirement for T<sub>reg</sub> to go for long periods in the periphery without TCR-ligation dependant survival signals. As activated T-cells are proposed to be the main source of IL-2, a reliance of T<sub>reg</sub> on an IL-2 dependant survival signal would enable the T<sub>reg</sub> compartment to mirror that of activated T-cells. Peripheral tolerance could therefore be maintained by the existence of a negative feedback loop in which activated T-cells and the T<sub>reg</sub> compartment are able to inhibit one another and

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therefore avoiding auto-immunity while maintaining an immune system which is fit for purpose.

We demonstrate IL-2 independent T<sub>reg</sub> development in TetZap70 FoxP3<sup>GFP</sup> mice treated with an IL-2 blocking antibody. Numerous studies describe an essential role for IL-2 in thymic de novo T<sub>reg</sub> development despite the observation that IL-2<sup>-/-</sup> mice still contain 50% of their T<sub>reg</sub> compartment (Vang et al. 2008). IL-2 is suggested to cause FoxP3 expression via STAT5 signalling, as the FoxP3 promoter contains a STAT5 binding motif. Indeed STAT5 deficient cells show defects in T<sub>reg</sub> frequencies, however studies in which the pro-survival molecule Bcl-2 is expressed in these cells, rescues the defect in T<sub>reg</sub> frequencies (Malin et al. 2009). This perhaps suggests an important role for IL-2 in facilitating survival of T<sub>reg</sub>. Further to this, we observed increased T<sub>reg</sub> percentages in mice treated with IL-2-Ab complexes. This data is consistent with studies in which a constitutively active form of STAT5 results in expansion of the T<sub>reg</sub> compartment in the absence of IL-2 (Yao et al. 2007). A more recent study in IL-2Rβ<sup>-/-</sup> mice demonstrates development of T<sub>reg</sub> with low expression of CD25 as well as low levels of the proliferation marker Ki67 (Cheng et al. 2013). It therefore remains unclear whether IL-2 functions to directly drive FoxP3 expression, induce changes in chromatin structure making cells more permissive to expression or promote survival and expansion. Our studies however show that FoxP3 expression can be induced in the absence of IL-2, and would seem to suggest a role for IL-2 in T<sub>reg</sub> survival and expansion is most

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likely. Reduced  $T_{reg}$  proliferation would successfully explain previous observations in  $IL-2^{-/-}$  mice in which the  $T_{reg}$  compartment is reduced by 50%.

### **What is the source of thymic IL-2?**

It is firmly established that IL-2 plays a pivotal role in peripheral tolerance through maintenance of the  $T_{reg}$  compartment. Although other  $\gamma c$  cytokines seem to play a semi-redundant role with IL-2, IL-2 appears the most crucial for  $T_{reg}$  differentiation/homeostasis. Previous studies have shown  $TCR^+$  T-cells secreting 'halos' of IL-2 within the thymus of IL-2 reporter mice (Yang-Snyder & E V Rothenberg 1998). It has therefore been proposed that paracrine IL-2 from a T-cell source is the primary source of IL-2 within the thymus. We present data suggesting a possible requirement for selecting T-cells in order to generate sufficient quantities of IL-2 to drive development of  $CD25^{Hi}$   $GFP^+$   $T_{reg}$  cells. Within TetZap70  $FoxP3^{GFP}$  mice injected with  $CD45.1^+$  mature T-cells, we observed only low levels of CD25 expression by thymic  $T_{reg}$  compartment (Fig 1.12), indicating that the presence of mature recirculant T-cells alone is insufficient to support  $CD25^{Hi}$   $T_{reg}$  development in the thymus. Conversely TetZap70  $FoxP3^{GFP}$  mixed chimeras which contain B1/6 bone marrow and therefore normal thymic selection, contain a prominent  $CD25^{Hi}$   $GFP^+$   $T_{reg}$  cell population (Fig C3.1/3.2). The observation that negatively selecting T-cells may be an important source of IL-2 in the thymus can be seen in TCR transgenic mixed bone marrow chimeras. It has been previously observed that TCR transgenic T-cells fail to undergo negative selection without thymic expression of cognate antigen (Ohashi et al. 1991). Therefore the observation that both OTI

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and OTII restricted mixed bone marrow chimeras were unable to support efficient CD25<sup>Hi</sup> GFP<sup>+</sup> T<sub>reg</sub> development may suggest a requirement for negative selection. This study therefore implies a requirement for efficient negative selection in order to drive thymic expression of IL-2 for T<sub>reg</sub> development/homeostasis. Perhaps suggesting an important link between the establishment of central and peripheral tolerance.

If true this hypothesis suggests that negative selection in TetZap70 FoxP3<sup>GFP</sup> mice generates low amounts of IL-2 relative to WT mice. In time courses of Zap70 induction with TetZap70 FoxP3<sup>GFP</sup> mice, we observed T<sub>reg</sub> emerging with low CD25 expression before CD25 levels gradually increase. This is perhaps characteristic of increased levels of thymic IL-2 as more cells enter selection. However, the proportion of T<sub>reg</sub> expressing high levels of CD25 never reached that observed in Bl/6 : TetZap70 FoxP3<sup>GFP</sup> mixed chimeras. This is perhaps indicative of impaired IL-2 secretion in TetZap70 FoxP3<sup>GFP</sup> mice, possibly due to sub-optimal TCR proximal signalling. It also remains possible that TetZap70 FoxP3<sup>GFP</sup> mice lack a fully mature thymic microenvironment and therefore the IL-2 secreting cell type normally present in WT mice.

A complete T-cell compartment has been shown to be crucial for the generation of a mature thymic microenvironment (Akiyama et al. 2008)(Hikosaka et al. 2008)(White et al. 2010). Un-induced TetZap70 FoxP3<sup>GFP</sup> mice lack a mature T-cell compartment and have therefore been shown to contain an immature thymic stroma (White et al. 2010). As a mature thymic medulla has been shown to be crucial for the development of FoxP3<sup>+</sup> T<sub>reg</sub>, it remains possible that

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development in TetZap70 FoxP3<sup>GFP</sup> mice remains inefficient (Cowan et al. 2013). DCs make up one of the many cell types contained within the thymic microenvironment and have been suggested to play an important role in thymic T<sub>reg</sub> development. Populations of DCs have been shown to migrate from the periphery to the thymus where they display peripheral antigens, supporting tolerance to peripherally expressed peptides (Proietto et al. 2008). DCs have also been shown to transiently express low levels of IL-2 (Granucci et al. 2001). A lack of thymic DCs contained within TetZap70 FoxP3<sup>GFP</sup> mice could therefore explain the low IL-2 levels observed within these mice. Although the low levels of IL-2 generated by thymic DCs may not be sufficient to generate large amounts of paracrine IL-2, local secretion whilst T<sub>reg</sub> interact with DCs during selection could be sufficient to drive CD25/FoxP3 expression. Previously within this study, we suggested a role for the trans-presentation of the IL-15R $\alpha$  by the thymic stroma in order to support T<sub>reg</sub> development by IL-15. Trans-presentation of CD25 has also been suggested to occur by some populations of DCs. This could suggest a possible mechanism in which DC-mediated IL-2 signalling could support FoxP3 expression in CD25<sup>Lo</sup> precursors cells (Wuest et al. 2011). If true and TetZap70 FoxP3<sup>GFP</sup> mice do indeed lack this DC cell population, this could explain the low levels of IL-2 activity observed in these mice.

### **What are the dynamics of de novo T<sub>reg</sub> development?**

The data in this thesis suggests a delay in the development of T<sub>reg</sub> relative to conventional CD4<sup>+</sup> SP cells. We show that T<sub>reg</sub> are first detectable 4 days after positive selection in the adult thymus, whereas conventional CD4<sup>+</sup> SPs were

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detectable by day 1. In addition to this we see the first wave of T<sub>reg</sub> developing with low CD25 expression levels. CD25 expression then increases, along with FoxP3 levels, as the cells develop further. Unexpectedly we demonstrate significant proportions of T<sub>reg</sub> recirculating back into the thymus from the periphery, suggesting the need for caution when interpreting data derived from thymocytes of lymphoreplete mice. The lack of this recirculant population of T-cells in TetZap70 FoxP3<sup>GFP</sup> mice goes some way to explaining the reduced thymic T<sub>reg</sub> frequencies in these mice when compared to WT animals. We further demonstrate a relative delay in thymic egress of T<sub>reg</sub> compared to conventional T-cell populations.

Our data appears to support observations made by previous studies which observe a delay in the development and egress of T<sub>reg</sub> compared to non-regulatory T-cell populations. However, why this delay exists remains poorly understood. The delay in FoxP3 induction compared to other lineage defining transcription factors has been the focus of numerous studies. This delay was previously observed in the first wave of T-cell development within neonatal mice. Delayed development of T<sub>reg</sub> compared to non-regulatory populations in these studies was suggested to be due to the lack of a cytokine signal (Fontenot et al. 2005). It was observed however that T<sub>reg</sub> in these mice express high levels of CD25, a phenotype we have shown to be characteristic of an environment rich in IL-2. The use of TetZap70 FoxP3<sup>GFP</sup> mice within our study has enabled the analysis of do novo T<sub>reg</sub> development in adult mice. We further confirm the previously described delay in development of T<sub>reg</sub> but conclude that



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this was not due to the lack of a cytokine signal. The delay in development could instead be characteristic of an extra round of differentiation and protein synthesis in addition to that already instructed upon commitment into the CD4<sup>+</sup> SP lineage, or perhaps due to a requirement for T<sub>reg</sub> to undergo multiple strong TCR-peptide signalling events in order to begin FoxP3 expression. Either scenario would explain the increased length of time T<sub>reg</sub> appear to spend developing in the thymus, prior to their delayed egress into the periphery. We show T<sub>reg</sub> present in the LNs of TetZap70 FoxP3<sup>GFP</sup> mice in substantial numbers 10 days after positive selection. This is a delay of approximately 5 days compared to that of non-regulatory CD4<sup>+</sup> SP populations. However it must be noted that extremely small numbers/percentages of cells coupled with large degrees of error mean care must be taken when interpreting this result. In fact small numbers of mice show evidence of T<sub>reg</sub> in the periphery as early as day 5, perhaps casting some doubt about the delayed egress of T<sub>reg</sub> compared to conventional CD4<sup>+</sup> SPs. More recent studies have also cast doubt over the precise timing of conventional and regulatory T cell egress from the thymus. In mice that have undergone day 3 neonatal thymectomy, they found significant proportions of FoxP3<sup>+</sup> T<sub>reg</sub> in spleen at early timepoints, yet mice succumb to auto-immunity (Samy et al. 2008; Dujardin et al. 2004). Whether this result illustrates the functional differences between peripherally derived iT<sub>reg</sub> and their inability to control autoimmunity when compared to thymic nT<sub>reg</sub>, that are missing in these mice, remains to be determined. However these studies would suggest there is a smaller difference in the dynamics of T<sub>reg</sub> development compared to conventional T-cells than perhaps previously thought.

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### **Alternative model for de novo T<sub>reg</sub> development**

According to the data presented in this thesis we propose an alternate linear model for thymic T<sub>reg</sub> differentiation. This model is composed of a TCR dependant phase, followed by a cytokine dependant phase for development. We suggest that strong early TCR/CD28 stimulation initiates FoxP3 gene transcription. It remains likely that histone modifications to the FoxP3 locus and regulatory region CNS1 precede FoxP3 expression. However acquisition of an activated chromatin state at these sites has been shown to be coincident with FoxP3 expression (Y. Zheng et al. 2010). Therefore it remains possible that chromatin modifications on alternative regulatory regions such as CNS3 play an important role in readying the FoxP3 locus for transcription. Following TCR/CD28 signalling, akt inactivation leads to enhanced FoxP3 expression via nuclear translocation of Foxo factors. This functions to further increase FoxP3 expression in the absence of TCR/CD28 selection signals. Multiple rounds of strong selection signals meanwhile lead to negative selection, while more infrequent signalling leads to FoxP3 expression. T<sub>reg</sub> precursors on the cusp of negative selection express CD25 prior to expressing FoxP3, while weaker selecting T<sub>reg</sub> precursors delay CD25 expression.

Following this, FoxP3<sup>+</sup> CD25<sup>Lo</sup> precursors continue to undergo multiple rounds of auditioning to become FoxP3<sup>+</sup> CD25<sup>Hi</sup> T<sub>reg</sub>. In the absence of a functional IL-2 receptor these cells rely on stroma derived IL-15 for survival as well as other  $\gamma$ c

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cytokines. If FoxP3<sup>+</sup> CD25<sup>Lo</sup> precursors increase CD25 expression following further rounds of high affinity selection signals they differentiate into FoxP3<sup>+</sup> CD25<sup>Hi</sup> T<sub>reg</sub>. If they fail the auditioning process they may lose FoxP3 expression and become exT<sub>reg</sub> or undergo negative selection. IL-2 secreted by negatively selecting T-cells in the thymus and activated T-cells in the periphery then stimulate proliferation of FoxP3<sup>+</sup> CD25<sup>Hi</sup> T<sub>reg</sub>, maintaining survival of the fittest T<sub>reg</sub> clones as well as controlling the size of the T<sub>reg</sub> compartment. This further enhances FoxP3 expression via STAT5 as well as enhancing expression of pro-survival factors.

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